

STUDIES ON PROTEINS OF GRAMINAE: SOME OF THEIR CHEMICAL
AND PHYSICAL PROPERTIES AND THEIR RELATIONSHIP
TO GENETICAL CHARACTERISTICS

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by

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PREFACE

Gluten, constituting the major protein fraction of wheat endosperm, is of importance commercially because of its unique visco-elastic properties. The aim of this investigation was to obtain some insight into the protein components comprising the gluten complex. This involved the development of a suitable method of protein fractionation, and its application to a study of the effects of physical, chemical and biochemical factors on the protein system.

The technique was also employed to follow the degradation and build-up of endosperm proteins throughout the life cycle of the wheat plant and in a comparative study of the proteins of ancient and modern wheats and the proteins of other Graminae.

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INTRODUCTION

(A) DETAILED CONSIDERATIONS OF WHEAT PROTEINS

1. Protein Distribution in the Wheat Seed

The anatomy of the wheat grain has been described by Percival (1921) in great detail. Basically, it consists of a single monocotyledonous seed enclosed within a thin-walled shell or pericarp. The seed proper may be conveniently regarded as consisting of the outer protective seed coat or testa which cannot be readily separated from the pericarp; the germ, consisting of the embryo and its sheath, the scutellum; the aleurone layer, which forms the outer part of the endosperm, and the endosperm or storage region. The nitrogen composition of these parts according to Hinton (1947) is given below:

	Proportion of the Grain %	Nitrogen Content %
Whole grain	-	1.40
Pericarp and testa	8.0	0.70
Aleurone layer	7.0	3.15
Embryo	0.94	5.33
Scutellum	1.5	4.27
Endosperm 1 (outer layer 150 μ in depth)	12.5	2.20
Endosperm 2 (2nd layer 150 μ in depth)	12.5	1.40
Endosperm 3 (inner layer 300 μ in depth)	57.5	1.00

These figures can conveniently be regarded as representing the approximate protein distribution in the seed. Although the aleurone layer and embryo have relatively high protein contents, the endosperm, which represents about 83% of/...

of the mass of the grain, must necessarily be regarded as the main site of protein material. The bulk of the proteins of the germ are thought to be enzymic in character. Information concerning the aleurone proteins is sparse, but they are thought to possess enzymic properties similar to those of the embryo.

2. Practical Importance of Gluten and Role in the Food Industry

The major significance of wheat flour in contrast to that from other cereals, is its ability to form a dough. During its preparation, the hydrated proteins aggregate to form the well-known gluten complex, which is responsible for the characteristic properties of the dough. Although flours from different species and varieties of wheat possess different physical characteristics, commercial fractionation procedures, judicious blending and often gluten supplementation, have allowed the preparation of the many grades of flour required by the baking industry. Modification by chemical means is however widely used, and the basis for many of these treatments is not yet fully understood.

Work of the geneticist has to some extent provided some control over the characteristics of individual flours, but a more fundamental knowledge of the gluten structure and individual species involved might lead to a better understanding of the factors involved in flour quality.

3. Historical and Modern Methods of Protein Isolation including Industrial and Laboratory Methods

Beccari (1745) first reported the isolation of gluten. By adding 60-65% water to a hard wheat flour, mixing and allowing the dough to age for about 30 minutes, then washing out the bulk of the starch and other soluble constituents in/...

in water, he obtained the elastomeric gluten mass containing about 70% water.

Basically, this is still the principal method of gluten isolation although individual modifications include pretreatment of the flour with organic solvents, sodium chloride solution or lignosulphonic acid (cf. Meredith et al., 1960a, Jones et al., 1959b; Pence et al., 1956; Ogawa et al., 1956a, 1956b).

Other methods of isolation include the direct extraction of the protein material from flour followed by the precipitation of the gluten from solution (cf. Jones et al., 1959b; Meredith et al., 1960a, 1960b; Cunningham et al., 1955).

Lusena (1950) described a laboratory method for the preparation of dried "purified" gluten by dispersing washed gluten in dilute acetic acid solution, centrifuging and freeze-drying the dispersion.

A more direct method of extraction is described by Hess (1953) who separated two morphologically different types of protein (adhering and wedge protein) from the endosperm by differential sedimentation in non-aqueous media.

Stevens et al. (1963) described a method for the isolation of interstitial endosperm proteins from flour based on the industrial air classification procedure mentioned below, combined with a technique involving differential centrifugation in non-aqueous media. This method also allowed the simultaneous separation of a fraction substantially rich in aleurone cell contents, from which the bulk of the starch was removed by further differential centrifugation.

Typical industrial processes for the separation of gluten include the so-called Martin process, based essentially on the technique of Beccari, (cf. Anderson et al., 1958) and the more recent "batter" process described by Dimler/...

Dimler (1944).

During the milling process, some of the endosperm cells are broken down into particles of various sizes (1 to 100 μ) (cf. Greer et al., 1951; Kent and Jones, 1952). Jones et al. (1959a) have shown that flour fractions separated on the basis of particle size contain different amounts of protein. Up to about 17 μ , the particles include pieces of free protein (cf. Hess, 1952, 1954) with a relatively high protein to starch ratio. Particles of 17-35 μ contain a large proportion of free starch granules and are consequently low in protein content. Above 35 μ , the particles consist of larger detached starch granules and pieces of unbroken endosperm cells, and may have a similar or higher protein content than that of the parent flour (cf. Jones, 1962). These differences have provided the basis for the industrial air classification of flours in the sub-sieve range (<40 μ).

4. Wheat Proteins and the Gluten Complex

Gluten, isolated by the method of Beccari (1745) contains about 85% protein, 8% lipid and 6% starch, the latter depending on the effectiveness of the washing procedure. As a result of the unique cohesive and elastic properties of gluten isolated by this procedure, there has been much speculation concerning its origin and its relationship to the native state of the proteins of wheat flour. There is considerable evidence based on the physical characteristics of isolated gluten to suggest that we are dealing with a 3-dimensional protein complex which is probably an artefact of isolation. Hence, before discussing the nature of gluten, it is necessary to define what are implied by the terms gluten and the gluten complex. Throughout this thesis wheat flour or endosperm proteins have been taken to/...

to refer to those native proteins extracted directly without the intermediate formation of a dough. Gluten proteins are regarded as those proteins extractable from gluten which has been isolated as a result of dough formation by the classical method of Beccari, or some modification of this technique and includes all proteins present irrespective of their solubility characteristics. The gluten complex is taken to refer to the gluten elastomer as isolated by the method of Beccari.

As a result of attempts to classify gluten proteins on the basis of solubility without consideration of the structure of the gluten complex, much conflicting information has been obtained with inevitable confusion over nomenclature (cf. Pence and Olcott, 1952; Pence et al., 1956; Pence and Mecham, 1962; Woychik et al., 1961b; Dimler and Senti, 1959; Mecham et al., 1962; Coates and Simmonds, 1961; Simmonds, 1963).

(a) Endosperm proteins

Hess (1953, 1954, 1955) believed that isolated gluten may not represent the conditions or composition of proteins in the original flour. He showed that flour and gluten proteins are very different in character, flour proteins existing in two forms, one as a network of fibrils on the surface of the starch granules, and covered by a layer of lipid ("adhering" protein) and the other as discrete particles between the starch granules ("wedge" protein). These two fractions can be readily separated by differential sedimentation in chloroform-ether or chloroform-benzene mixtures, and have been shown to differ greatly from the gluten separated from flour by water. They produced different X-ray diffraction patterns before and after swelling in water and had slightly different isoelectric points (cf. Pence et al., 1956). Hess concluded that during the conversion of native protein/...

protein to gluten, mechanical treatments cause a structural chemical alteration of the protein molecules.

Recent results obtained by Stevens et al. (1963) based on amino acid analysis indicated that proteins of the "gliadin/glutenin" type are preferentially extracted in flour fractions of small particle size ($<17\mu$) during air classification, while the more soluble protein fractions appear to be associated with those fractions of larger particle size containing more starch (cf. Wrigley, 1963). Moreover, the similarities in amino acid composition between some of the latter fractions and the isolated aleurone proteins indicated that these may possibly originate to some extent in the aleurone cells.

Recent work by Graham et al. (1962) has shown the presence of "protein bodies" in the endosperm of the developing grain. Electron microscopic studies showed that these bodies, containing about 85% protein, are surrounded by a lipoprotein membrane and appear to be the protein storage region of the developing seed. Similar protein bodies have been observed in maize, sorghum and peanut (cf. Duvick, 1955; Watson et al., 1955; Altschul et al., 1961).

(b) The gluten complex

Much of the work on wheat proteins has been based on the investigation of the proteins of the isolated gluten complex, since because of its unique cohesive properties it is readily separated from most non-nitrogenous constituents thereby overcoming a major problem in the study of plant proteins. It has already been pointed out however that there are inherent dangers in drawing conclusions concerning flour proteins as a whole when one is dealing with isolated gluten as a/...

a starting material.

Pence and Mecham (1962) state that roughly 80% of the protein material in wheat flours comprises the complex termed gluten. Of the remaining 20% approximately half is accounted for by each of the two classes of components that seem properly designated as albumins and globulins. They further define gluten as the complex formed of about equal parts of gliadin, the fraction soluble in aqueous alcohol, and glutenin, the fraction insoluble in alcohol. In earlier papers however (Pence and Olcott, 1952; Pence et al., 1954a) it is pointed out that gliadin occurs to a significant extent in water or dilute salt extracts of flour, and so tends to obscure effects associated with the soluble "non-gluten" proteins. The importance of these "non-gluten" proteins must be considered since some are believed to be enzymic in character, while others may substantially modify gluten behaviour, even if they may not be considered structural materials in the protein framework of bread. They are essential in the reconstitution of doughs and without them the physical properties of gluten are altered (Pence et al., 1956). Moreover, vigorous mechanical washing to give a gluten practically free of starch fails to remove more than about half of the soluble proteins, suggesting that they may be bound to the gluten during dough formation. Similarly Dimler and Senti (1959) suggest that the albumins and globulins of wheat flour may either be trapped in the gluten mass or form loose complexes with the gluten proteins.

Meredith et al. (1960a, 1960b) propose that gluten consists of a complex structure composed of a gel-protein network with proteins and other fractions, including possibly lipids, bound to it in various ways. Dilute acids and probably alcohol/...

alcohol and other reagents easily cause splitting of the complex. This implies that with most dispersions of gluten, one is dealing with mere fragments of the original gluten complex, the amounts of which will depend on the conditions of dispersion. This view is also held by Mecham et al. (1962) who found that when repeatedly extracted with dilute acid, freeze-dried flour-water doughs yielded more protein than the flour from which they were prepared. The additional extractable protein appeared to be derived largely from the material which in suspensions of the flour settled rapidly and was highly hydrated. It does not seem justified then to dismiss those proteins which are partially soluble in water or dilute salt solutions as "non-gluten" or "contaminant" proteins (cf. Simmonds, 1962), since there is much evidence to suggest that they may form an integral part of the gluten complex and may themselves be artefacts of isolation, or exist in equilibrium with the more firmly bound species.

A somewhat different view is held by Axford and Elton (1960) and Axford et al. (1962) who favour the theory that the 3-dimensional network of gluten forms as a result of the formation of covalent disulphide bonds between cysteine residues in the protein chains. Hydrogen bonding also contributes significantly at low temperatures. They propose the rupture of intra- and inter-molecular disulphide bonds as a result of mechanical action on the dough. The free radicals which occur would recombine to form new disulphide links or react with other components to give rise to cross-linkage and an expandable 3-dimensional network.

The microphysical structure of gluten was examined by Grosskreutz (1960) by means of small-angle X-ray diffraction and electron microscopy. The/...

The proteins in wet or dry isolated gluten appeared to consist of folded polypeptide chains in the α -1-helix conformation, arranged in flat platelets of the order of 70 Å units thick. Such platelets were postulated to form sheets capable of large plastic deformations as long as phospholipid structures, appearing to exist in the form of oriented bimolecular leaflets, are present to furnish slip-planes in the overall structure. In a later paper Grosskreutz (1961a) found that gliadin in solution also tends to exist in sheet form, although specimens prepared for electron micrography exhibited a doughnut shape. Glutenin under similar conditions showed an oblate ellipsoidal shape (Grosskreutz, 1961b).

These views are possibly open to doubt since X-ray and electron micrography especially may be revealing artefacts of slide preparation (Spurling, 1961).

A similar investigation was reported by Reed and Weston (1962) who reported that alcoholic solutions of crude gliadin contained spherical particles ranging from 100 Å to 250 Å in diameter. These appeared to form filaments by linear aggregation, which in turn could associate laterally to form broad fibres. Branching of these fibres produced complex networks which closely resembled the structure of whole gluten.

Isolated gluten has been shown to contain about 8% lipid material (Sullivan, 1954). At least some of this appears to be combined with the protein in a manner typical of lipoproteins in that alcohol or similar solvents are necessary to extract them. Olcott and Mecham (1947) proposed the term "lipo-glutenin" for that fraction of protein binding most lipid. Reports by several other workers (cf. Sullivan, 1954; Pence et al., 1956; Hess, 1954; Zentner, 1960; Mecham and/...

and Pence, 1957) also seem to confirm the presence of protein-bound lipid in isolated gluten.

Despite this however the pretreatment of flours to remove lipids before gluten extraction has been carried out by many workers (Meredith et al., 1960a; Jones et al., 1959b; Pence et al., 1956). This "defatting" procedure may actually split naturally occurring protein-lipid bonds and result in the extraction of a modified gluten (cf. Mecham and Mohammad, 1955; Bhatti and McCalla, 1958).

It has been shown (Pace, 1959) that 70-80% of the lipid material may be extracted from flour with ethyl ether or light petroleum, but alcohol or other solvent capable of dissociating lipid-protein bonds is required to remove the remainder. On dough formation, however, 70-90% of the lipid is made^{un}extractable in ethyl ether. There is also evidence (Olcott and Mecham, 1947) to suggest the presence of phospholipids which are more firmly bound to the complex than the glyceride portion of the fats.

Traub et al., (1957) found that in X-ray studies, wheat grain sections exhibit a spacing of 47 \AA units not observed in other cereals, which they attributed to that fraction of the fat associated with the gluten proteins, particularly with the phospholipid fractions. They suggested that the protein fibres may be held together by layers of phospholipid with the fat molecules roughly perpendicular to the protein fibres.

(c) Solubility behaviour

Much of the classical work relating to the isolation of protein fractions from wheat flour or gluten is based on solubility methods. Osborne (1907) first separated the proteins of flour into 5 main fractions: gliadin, a prolamine soluble in/...

in 70% ethanol; glutenin, soluble in dilute acid or alkali; a neutral salt-soluble globulin; a water-soluble, heat coagulable albumin; and an ill-defined proteose. This separation has subsequently been used as a basis for preparing crude fractions of gluten proteins. It has since been shown however that solubility criteria cannot be relied upon as a method of obtaining "pure" fractions of gluten proteins (cf. Pence and Olcott, 1952; Pence et al., 1954a) since one is not dealing with true solubility effects, but rather a 3-dimensional protein complex, the structure of which is greatly dependent on its method of isolation, mechanical treatment and its ionic environment (see also Gortner et al., 1929; Blish and Sandstedt, 1929; Sandstedt and Blish, 1933; McCalla and Gralen, 1940, 1942; McCalla and Rose, 1935; Rich, 1936). Pence and Olcott (1952) for example found that the solubility of albumins and globulins and gliadin are all influenced by salts present in the extracting solutions, while Kaminski (1962) (cf. Coulson and Sim, 1961; Elton and Ewart, 1962), following the extraction procedure of Osborne, found that there was a considerable overlap in the protein fractions when these were compared by electrophoresis in starch gel. This is also emphasised by the work of Pence and Olcott (1952), Pence et al. (1954a, 1956) and Dimler and Senti (1959) already mentioned.

The effects of certain solvents on the gluten complex have already been discussed in terms of the splitting of lipid protein complexes (cf. Bhatti and McCalla, 1958; Meredith, 1961). Dilute acids are also thought to be effective agents in the breakdown of the gluten complex (Meredith, 1960). Moreover the glutenin fraction is taken by many to represent the denatured residue resulting from the alcoholic extraction of gliadin (cf. Blish, 1945) and is regarded as/...

as a derived rather than a native protein (Blish and Sandstedt, 1926). Pence and Mecham (1962) however found that when gliadin is removed from crude gluten by methods which avoid the use of alcohol, the glutenin remaining is even more elastic and extensible than the original gluten. They suggested that the honey-like consistency of gliadin might indicate that it is a lubricant, plasticiser or inert diluent of the glutenin. The effect of mechanical treatment of the gluten during the extraction procedure must also be considered since the gluten complex is obviously a work-sensitive system (cf. Mecham et al., 1962).

(d) Biochemical characteristics of wheat proteins

Much of the work concerning the enzymic characteristics of wheat proteins seems to be confined to those enzymes which appear to effect the rheological characteristics of doughs and hence the baking quality of flour. There are few reports of a fundamental nature concerning these systems but with new methods of protein fractionation, particularly on a preparative scale, and with the increasing interest in the biochemical nature of wheat proteins especially in the more soluble fractions, further information is likely to be forthcoming in the near future.

As early as 1884 Balland (cf. Johnson et al., 1929) observed the presence of proteolytic enzymes in extracts of germinating wheat seeds which, when incorporated in doughs caused rapid degradation of the gluten. Mounfield (1936) and Balls and Hale (1938) reported a proteinase in wheat seeds, which was purified by Hale (1939) and shown to have an activity of about 1/400 of that of papain. These enzyme preparations were later shown (Danielsson, 1951a) to contain at least two proteolytic enzymes, one proteinase, with its optimum activity at pH 4.1/...

pH 4.1 and one dipeptidase with optimum activity at pH 8.5.

Jørgensen (1935a, 1935b, 1936a) proposed that the proteolytic enzymes of wheat flour belong to the papain group. Moreover, he suggested that the improving effects of oxidising agents (e.g. bromates and iodates) on dough is due to an inhibition of those enzymes inherent in the wheat flour, although by partial hydrolysis of the peptide chains of gluten their effect would be useful in causing a stiff gluten to become more pliable and elastic. It was pointed out by Balls and Hale (1936) however that if these enzymes are present in large amounts they may result in the production of amino acids and so cause a deterioration of the baking quality of the flour (cf. Bell, 1963). Similarly, the deteriorating effect of reducing agents (e.g. glutathione and cysteine) would be due to an activation of these enzymes.

This view is also held by Bloksma (1958) and Fajersson (1961), although they point out that the effects of redox systems on dough are probably only partly explained by their effect on proteolytic enzymes. The direct effect of redox systems on gluten proteins will be discussed in a later section.

A detailed examination of the enzyme activity of the developing wheat grain is reported by Rijven and Cohen (1961) and Rijven and Banbury (1960). They found a very high transaminase activity in the endosperm of the grain. Glutamyl transferase activity was found almost exclusively in the future grain coat tissues, showing a peak of activity early in the development cycle. Acid phosphatase activity was found in all tissues, with highest specific activity in the regions of earliest breakdown. The embryo was shown to contain a high activity for alcohol dehydrogenase.

Elton and Ewart (1962) observed the effects of β -amylase activity after electrophoresis of flour extracts in starch gel, while Pence, 1953 (cf. Pence et al., 1956; Pence and Mecham, 1962) suggested that the α -2-albumin component as separated by free boundary electrophoresis is β -amylase.

In a recent paper, Rowsell and Goad (1962) reported that about 80% of the β -amylase of wheat flour was of an inactive latent form which could be released by the action of proteolytic enzymes (e.g. papain) when activated by cysteine or by cysteine alone. They concluded that the latent β -amylase was chemically bound to the glutenin fraction while the active β -amylase was confined to the soluble albumin-globulin fractions (cf. Pence et al., 1956). The binding of β -amylase to glutenin was thought to be by disulphide linkages and could be released by splitting these bonds or by the splitting of a peptide bond to release a peptide- β -amylase fragment. Embryos were found to secrete a substance which releases glutenin-bound β -amylase.

Although the embryo is generally considered to contain a large number of enzymes (cf. Bondi, 1958), which probably initiate the breakdown of protein during germination, few reports of work in this field are available. This is presumably due to the bias in interest towards those proteins more intimately concerned with problems of rheology.

(e) Biophysical characteristics of wheat proteins

(i) Stability. Approximately 2/3 of the weight of wet crude gluten is accounted for by water of hydration and when dehydrated by drying at low temperatures under vacuum, it may be reconstituted without apparent loss in its physical or/...

or colloidal properties (Blish, 1945). Blish and Sandstedt (1926) found, however, that prolonged drying at 60-65° in a partial vacuum resulted in almost complete denaturation of glutenin, without significant effect on gliadin (cf. Sharp and Gortner, 1923). Olcott and Blish (1944) showed that gluten is rapidly denatured by drying at atmospheric pressure and especially so after boiling in water. Moreover they found that the solubility of gluten in dilute acetic acid solution and alcohol gave a measure of the degree of denaturation. With certain modifications, principally the elimination of alcohol, this test is still in current use in the baking industry (cf. Schaefer, et al., 1960).

In contrast to the extreme lability of gluten to heat especially when hydrated, many workers have found that dispersions of gluten in dilute acids are usually stable. Olcott et al. (1943) reported that such solutions may be heated at temperatures of 95-100° without significant alteration of the protein components. Gluten solutions are commonly heated to such temperatures (to inactivate proteolytic enzymes) before fractionation by electrophoresis (cf. Jones et al., 1959b; Woychik et al., 1961b). Buffer systems including concentrations of urea up to 3 M have also been used in these experiments without significant alteration of the protein components other than a compacting of the migrating species.

Blish (1945) proposed that the unusual stability of gluten proteins to conventional denaturing agents may be explained in terms of molecular structure of the native proteins, since there is some evidence to suggest that they are elongated or rod-like rather than essentially spherical (cf. Neurath, 1939) and so do not undergo the conventional molecular "unfolding" of globular proteins.

(11)/...

(ii) Rheological properties. The unique importance of wheat flour in the baking industry is a result of its ability to form doughs which possess elastic, viscous and plastic properties. These properties are generally accepted as being attributable to the gluten proteins and closely associated substances in the flour, since, on removal, the gluten mass exhibits very similar physical characteristics. Flours of different sources and varieties of wheat, but of equal grade and protein content may however display wide variations in the physical properties of their doughs and glutens, which have been attributed to some extent to genetic characteristics, but as yet no completely satisfactory explanation has been obtained to account for them.

There have been many attempts to explain the physical characteristics of gluten in terms of the amounts of gliadin, gluten and water-salt soluble fractions. Fleurent (1896) believed that the larger the amount of gliadin, the more extensible was the gluten. More recent work has shown, however, that both these fractions form an integral part of the whole gluten complex and so are intimately connected with its physical characteristics.

There is however a considerable amount of evidence to suggest that the more soluble albumin-globulin fractions contribute significantly to the physical characteristics of doughs (cf. Finney, 1943). Pence et al., (1954b) showed that both albumin and globulin contents as well as the ratio of albumin to globulin varied significantly in different flours. They also found a correlation between the albumin-globulin ratios and the baking quality of the flours. The baking properties of crude glutens obtained from flours of widely varying properties were rather similar when the "soluble"/...

"soluble" fractions were omitted from the reconstituted doughs. Original differences in quality were apparently restored however, when these soluble materials were added back to the gluten-starch doughs (cf. Pence et al., 1951).

It would seem then that the "soluble" proteins play an important role in the physical characteristics of doughs and hence they must be considered part of the dough or gluten complex when dealing with rheological properties.

(iii) Mechanical effects. There is much evidence to suggest that the gluten complex is a work-sensitive system.

Skovholt (1935) found that overmixing of a dough resulted in drastic alterations in the physical properties, which was attributed to the division of protein aggregates rather than to the actual splitting of proteins (cf. Axford and Elton 1960). Halton and Scott-Blair (1937) showed that increased stress on doughs causes a fall in viscosity, while increased strain causes an increase in viscosity. Dempster et al. (1953, 1954) found that the internal stress of a dough diminishes during rest. Udy (1953) however, found that "purified" gluteins become more resistant to stretching after resting, as contrasted to doughs which soften during resting.

These changes appear to be dependent on the amount of work applied to the system, and may be explained in more fundamental terms of bond splitting, either covalent, hydrogen or electrostatic. The possibility of this will be reviewed later in this section.

(iv) Irradiation effects. Ultrasonic irradiation has frequently been employed in the disintegration of tissue cells and as a useful method of dispersion. It has also been shown to cause significant alteration of macromolecules (cf. El'piner, 1960/...

1960, Weissler, 1960) resulting in degradation and in some cases polymerisation. Changes in enzyme characteristics have also been observed.

Although this treatment does not seem to have been applied either to the dispersion or alteration of wheat proteins, its effect might produce interesting changes which could be potentially useful in the modification of the work-sensitive gluten complex.

γ -irradiation has however been shown to have some improving effects on flour doughs. Deschreider (1961) showed that irradiation of lyophilised gluten caused significant molecular degradation as well as a certain degree of polymerisation. Irradiated flours were shown to have better baking values attributed to a reduction of free -SH groups and to the formation of disulphide bonds. A similar improving effect was reported by Doguchi et al. (1959) at radiation levels of 13×10^4 to 1067×10^4 rad, without apparent alteration of the protein-paper electrophoresis pattern. Vakar and Tolchinskaya (1960) however reported an increase in dough elasticity at levels of 0.5 to 1 M rad, while larger doses (approx. 12 M rad) had an improving or stiffening effect on the dough.

(v) Biochemical effects. The theory proposed by Jorgensen (1935a) that the effects of redox systems on flours may be accounted for by inhibition or activation of papain-like enzymes, has already been mentioned in the section dealing with the biochemical properties of gluten. Much of the evidence in support of this theory is based on the assumption that the proteolytic enzymes of wheat are papain-like in character, and on the fact that most of the factors which affect dough and gluten characteristics do have an inhibiting or activating effect on papain. Although/...

Although papain-like proteinases have been isolated from wheat bran by Balls and Hale (1938) and also from flour by Hale (1939), several workers have reported the activation or inhibition of papain by reagents which have no such effect on wheat proteinases (cf. Read and Hass, 1937; Hites et al., 1953). Moreover, Hites et al. (1951) report the presence of a papain inhibitor in flour and especially in bran, which does not inhibit the proteolysis of haemoglobin by flour proteinases, (cf. Johnson and Miller, 1953). Also, the optimum pH for the hydrolysis of gluten by papain differs from that for the hydrolysis by malted wheat proteinase (Johnson et al., 1956). Fractions obtained from enzyme treated doughs by precipitation from sodium salicylate solutions have shown that the action of flour proteinases and papain are very different. It would seem then that flour proteinases and papain are not identical,

Olcott et al. (1943) reported the presence of a heat sensitive proteolytic enzyme in gluten, which is activated to some extent by reducing agents. After heating, however, the gluten was still susceptible to the action of reducing agents.

(vi) Chemical effects. Although heating appears to reduce the effect of redox systems on gluten to some extent, it still remains susceptible to a large degree. This would seem to suggest that the effect of these reagents, although partly explained perhaps in terms of enzyme activation and inhibition, is largely a direct action on the individual protein components which comprise the gluten complex.

Sullivan (1954) points out that the chemically reactive groups which would seem to be involved in the changes in the physical properties of gluten and dough on oxidation and reduction belong to the sulphur containing amino acids, methionine, cysteine and cystine.

There is considerable evidence to suggest the involvement of these groups in the physical characteristics of gluten. Miller et al. (1950) found a correlation between the cystine contents of hard red winter wheats and mixing times of flour derived from these wheats, while Wostmann (1950) reported a correlation between cystine content and resistance to extension in doughs as measured by the area under extensograms.

Cystine is considered to be the source of numerous disulphide linkages between polypeptide chains and hence is at least partly responsible for the integrity of the gluten structure. The effect of reducing agents, especially thiol compounds, on gluten is considered by many to be due to a rupture of these disulphide bonds with a resultant increase in extensibility and softness of the gluten (cf. Sullivan, 1954; Pence et al., 1956; Pace, 1959). The percentage of cystine involved in this interchain linkage is still unknown and as pointed out by Sullivan (1954) some of the cystine may be involved only in single chains (cf. Pace, 1959).

Very small quantities of oxidising agents such as potassium bromate and iodate (10-60 parts per million) and also oxygen, produce relatively large changes in the gluten properties, making it more elastic, cohesive and less extensible. Although the total extent of these changes does not correlate satisfactorily with the amount of oxidant required, it is generally considered to be an involvement of the free sulphydryl groups of cysteine. Due to the low level of reducing activity of gluten however, it is difficult to assess accurately the degree to which these groups are involved.

Sullivan (1954) proposed that when a dough is worked in the presence of oxidising agents, oxidised sulphydryl groups in close proximity are realigned by the shearing/...

shearing force to form disulphide cross-linkages which produce a toughening of the gluten. It is also pointed out by Pace (1959) that small molecular weight substances containing free sulphydryl groups, e.g. glutathione (potential reducing agents of disulphide linkages) will be similarly oxidised.

Mechem (1959) has shown that the physical properties of doughs can be markedly altered by the addition of specific sulphydryl blocking reagents such as N-ethylmaleimide (NEMI), p-chloromercuribenzoate (PCMB) and iodoacetamide during mixing in amounts roughly equivalent to the sulphydryl content of the flour. Under these conditions, the reagents produce an improving (stiffening) effect on the dough. Excess of NEMI, however, was shown by Meredith and Bushuk (1962) and Bushuk and Hlynka (1962) to have a deleterious effect, suggesting a breakdown of the dough structure by some mechanism other than by reaction with free sulphydryl groups. Similarly, oxygen was shown to cause an initial improving effect, but its effect on doughs with relatively few or no free sulphydryl groups was similar to that with excess NEMI, but to a lesser extent. It was proposed that this second, much slower reaction may involve the breakdown of disulphide bonds.

It seems probable that the lipid material associated with the gluten also plays some role in the rheological properties of doughs. Oxidising agents have been shown to affect flour lipids to some extent, probably by peroxidation of the unsaturated fatty acids, possibly leading, according to Pace (1959), to some degree of polymerisation or direct non-enzymatic transfer of oxygen to the proteins (cf. Morrison, 1963). This view is also expressed in recent work by Tsen and Hlynka (1962) who proposed a three-phase mechanism whereby the lipids compete with/...

with free sulphydryl groups for the available oxygen during dough mixing, resulting in the formation of lipid peroxides. These in turn react with sulphydryl groups to produce disulphide linkages. The third phase involves the direct oxidation of sulphydryl to disulphide mentioned above. Moreover, Naryanan and Hlynka (1962) have shown that doughs from defatted flour are more sensitive to oxidising effects indicating that lipids have a protective action against the improving effect of oxygen (cf. Rohrllich, 1962b). Similar results were considered by Bungenberg de Jong and Klaar (1952) to indicate the unimportance of lipids in dough oxidation, but in view of more recent findings this interpretation would seem unlikely (cf. Ponte et al., 1963).

(vii) Amino acids and hydrogen bonding. Attempts to explain the unique physical characteristics of gluten in terms of amino acid content have been relatively unsuccessful.

Pence et al. (1950) found that glutens obtained from flours of different dough characteristics and baking qualities have no apparent variation in the kinds or amounts of amino acids present. Significant differences in amino acid content of individual protein fractions have however been shown by Woychik et al. (1961a) who proposed that the high levels of glutamic acid and proline which together account for about half of the peptide bound amino acids, are probably indicative of their structural importance in gluten.

Bloksma (1958) reiterates this view and proposes a formation of hydrogen bonding through acid amide groups. By examination of spread monolayers of gluten, Tschoegl and Alexander (1960) similarly concluded that the unusually strong/...

strong cohesion of gluten films was attributable to hydrogen bonding through the numerous amide groups on glutamic acid side chains.

(viii) Electrostatic bonding. Bungenberg de Jong and Klaar (1952) also proposed the existence of an electrostatic attraction between gliadin and glutenin fractions provided that the pH is between the isoelectric points 5.4 and 6.5. Similarly, Hess (1955) suggests an electrostatic attraction between wedge and adhering proteins during their combination in dough formation.

(f) Factors associated with the gluten complex

Frazer et al. (1959) and Schneider et al. (1960a, 1960b) showed that gluten is pathogenic in coeliac disease and idiopathic steatorrhoea. It has been shown to contain a water soluble, heat stable substance which depresses the peristaltic reflex of an isolated strip of intestine. The isolation and identification of this active factor, thought to be a peptide associated with the gluten, is of major importance, because of the wide use of flour, gluten and associated substances derived from it (e.g. monosodium glutamate) in present day foodstuffs.

No reports appear to be available on electrophoretic or amino acid analysis of this substance, both of which techniques would provide a basis for comparison with the many similar analyses of gluten fractions reported in the literature,

The presence of a polysaccharide-protein fraction in wheat flour has also been reported by Bell (1963) and Rohrllich (1962a).

5. Wheat Life Cycle

(a) Germination

Much of the early work concerning the changes which occur in the protein constituents/...

constituents of seeds during germination has been reviewed by Chibnall (1939).

It is well known that germination involves the metabolism of reserve proteins of the seed, which are hydrolysed to amino acids and amides for subsequent transportation to the growing tissues of the seedling (cf. Bondi, 1958). Comparison of the amino acid composition of the reserve proteins and those in the growing embryonic regions, indicates that the germination must involve not only a protein breakdown, but also a resynthesis in the growing tissues (cf. Bonner, 1950).

Amides have also been shown to accumulate in the seedling during germination, usually in large excess of the amount available through protein hydrolysis in the parent seed. The accumulation of large quantities of asparagine and to a lesser extent glutamine, was noted by Schulze in his early work. This amide formation is thought to represent a detoxification mechanism for the removal of ammonia formed by deamination of amino acids during proteolysis.

Most of the early work on germination has been confined to a study of degradation products rather than the actual function of the reserve proteins involved. With the advent of newer methods of protein fractionation however, a clearer picture of the role of different protein species in the plant has been obtained, and this will inevitably lead to a clearer understanding of such mechanisms as germination and seed maturation.

Few reports are available concerning the germination of wheat seeds, although some information may be obtained from the work of Danielsson (1951b), who examined the amount of unaltered proteins present in pea seeds at different stages of germination. By ultracentrifuge methods, he showed that the breakdown of/...

of reserve proteins, especially globulins, is greater after 5 to 10 days germination. This maximum was found to coincide with the formation of leaves in the growing plant, thus suggesting the utilisation of these components for re-synthesis of proteins in the new leaves. In contrast, the albumin proteins appeared to be degraded at a slower, more constant rate.

Some of the changes in the protein constituents of germinating peanuts were reported by Dechary et al. (1961). Changes in protein constituents were followed by column chromatography fractionation and electrophoresis in polyacrylamide gels. This allowed the isolation of one of these fractions, the globulin, α -conarachin, which appeared to be broken down early during germination. It was proposed that a more detailed study of the metabolism of this and other fractions may throw some light on the nature of the true reserve proteins.

(b) Seed maturation

One of the few investigations on the synthesis of reserve proteins in seeds was reported by Danielsson (1952), who examined the formation of the reserve globulin proteins, vicilin and legumin, and the albumin proteins during ripening of pea seeds. By ultracentrifuge methods, he showed that the high molecular weight globulins are synthesised rapidly during the first half of the ripening period, while the albumin content increases slowly at a constant rate. Curves for the formation of protein nitrogen, globulin nitrogen and albumin nitrogen were very similar to those obtained for their breakdown during germination, except that the initial formation of these well defined components was characteristically rapid. Examination of the components of harvested unripe seeds, showed that the ripening process/...

process continues even after removal from the mother plant. By comparison with the amounts of amino nitrogen present in these seeds before and after formation of reserve proteins, it was concluded that synthesis occurred by condensation of large molecular weight peptides, rather than by a gradual build-up from the available amino acids. Moreover, this seemed to be substantiated by the rate of formation of these components.

Graham et al. (1962) reported an investigation of protein synthesis in ripening wheat seeds by examination of endosperm sections in the electron microscope. Results indicated an initial period of rapid synthesis of protein material during seed maturation, similar to that observed by Danielsson. This occurred particularly within enclosed protein bodies in the endosperm. Examination of these proteins by starch gel electrophoresis showed that they closely resembled the slow moving components extracted in acetic acid from intact endosperm. These components, which were shown to increase quantitatively rather than qualitatively during ripening, are thought to represent the storage proteins of the endosperm.

An examination of the peptides present in ripening wheat and rye from shortly before ripening until harvest was reported by Rohrllich (1962a). Water-soluble extracts were shown by column chromatographic procedures to contain a characteristic series of neutral, acidic and basic peptides consisting of two to twenty amino acids. These were shown to decrease in amount during ripening. Water soluble extracts of wheat and rye flour were also shown to contain peptides.

6. Genetical Influences on Endosperm Proteins

Attempts to explain the characteristic differences in the physical properties

of/...

of flour from different types of wheat have so far been relatively unsuccessful. It is generally accepted that these differences are inherent in the protein components, and are probably attributable, at least in part, to the influence of genetic factors introduced during the development of the species.

The recent development of new techniques of protein fractionation has shown that distinct differences indeed exist between the protein components of different types of wheat. A correlation of these differences with the characteristic properties of flour has as yet not been possible, but this may be due in part to the close genetic relationship between the species investigated.

Laws and France (1948) reported a comparison of the acetic acid and water-soluble proteins of different varieties of vulgare or bread wheats by free boundary electrophoresis. Although the flours investigated showed marked differences in physical characteristics, no significant differences were obtained in their protein patterns.

Pence et al. (1954b, 1954c) however reported significant differences in the albumin and globulin fractions of flour from different wheat varieties. Marked quantitative differences were observed between the different species, durum, compactum and vulgare, while only minor differences existed between varieties of common wheat. Similar results were reported by Kelley and Koenig (1963).

Simmonds (1963) reported significant qualitative differences in the pyrophosphate soluble components of different flours, as judged by column chromatographic fractionation on DEAE-cellulose.

Using a salting-out method, Tiunova (1960) reported only small differences between/...

between the protein components of gliadin from a common vulgare wheat and its parent forms including couch grass. Significant quantitative differences were however observed in the albumin and globulin fractions of these wheats, which could be related to flour characteristics (Tiunova, 1961a).

Elton and Ewart (1962) reported significant qualitative and quantitative differences in the slow-moving components of different varieties of common commercial wheats as judged by starch gel electrophoresis. No significant differences were however reported in the albumin and globulin fractions. Marked differences were also observed in the protein components of related gluten-producing cereals. Although no attempt was made to correlate these differences with flour characteristics, it was recognised that a comparative examination of this kind may give some information concerning those proteins responsible for the characteristic properties of gluten.

(B) ANALYTICAL STUDIES ON WHEAT PROTEINS

1. Amino Acid Composition and Supplementation

Osborne and his coworkers were first to undertake extensive amino-acid analysis of "purified" samples of gliadin and glutenin. Although he was able to account for more than half of the amino acids present in these preparations, an exact and detailed analysis was not possible until the development in recent years of improved and refined techniques.

The total amino acid composition of crude gluten has been extensively investigated, and the usual amino acids have been shown to account reasonably satisfactorily for the nitrogen present (cf. Pence et al., 1956). The outstanding feature of/...

of gluten proteins is the extraordinarily high content of glutamic acid, especially in gliadin, where it constitutes nearly half of the entire protein substance. There also exists a significantly large proline content, but relatively small amounts of basic amino acids and tryptophan.

An extensive amino acid analysis of gluten proteins and wheat protein fractions isolated by chromatographic procedures, was recently reported by Woychik et al. (1961a). Characteristic differences were observed between the amino acid composition of the water solubles and other isolated gluten fractions, particularly in the content of basic and carboxylic acids. Sufficient ammonia was present to account for over 90% of the dicarboxylic acids as their amides. Glutamic acid and proline together accounted for half or more of the total peptide-bound amino acids of the gliadin fractions, thus indicating their structural importance in gluten.

The possible role of cystine and cysteine in relation to the physical properties of the gluten complex has already been reviewed.

The total amino acid contents of crude glutens from a wide variety of flours were shown by Pence et al. (1950) to be surprisingly uniform, and these failed to reflect the significant differences in dough characteristics. It was pointed out that this may have been due to the standardised procedure of extraction, and did not include the varying amounts of soluble proteins present in the flours. McElroy et al. (1949) and Price (1950) however both found significant variation in several amino acids of whole wheats, which correlated with the total protein present. This was interpreted by Price to indicate varying ratios of the different protein components. A similar conclusion was reached by McDermott and Pace (1960) and Simmonds (1962)/...

(1962) who reported a significant variation in the lysine and arginine contents of flours of different baking characteristics. They found that increased protein content was accompanied by a decreased lysine and increased glutamic acid content.

Stevens et al., (1963) reported differences in the overall amino acid composition of the protein material of a flour fraction (particles $<17\mu$) isolated by the air classification procedure, when compared with that from the parent flour. A higher glutamic acid and proline content and a lower aspartic acid, lysine and arginine content, were taken to indicate the possible segregation of "gliadin/glutenin" type proteins during the flour fractionation, and a preferential association of "soluble" protein with those fractions of higher starch content. Similar results were obtained for samples of weak and strong flours. The amino acid composition of aleurone proteins was found to differ significantly from that of the main gluten proteins. The relatively high arginine content suggested some similarity to the more soluble flour fractions.

The factors of environment and fertilisation have been shown to exert important influences on the amino acid composition of wheat proteins. In fact Hussein (1961) reported that environment was more important than variety in determining the amino acid composition of gluten and its water soluble fractions. Miller et al., (1950) found significant variation in cystine and methionine contents of wheats grown in different environments and in different years. Similarly, Volker (1960) reported a decrease in the proportion of lysine and arginine with a corresponding increase in glutamic acid on fertilisation. This is in agreement with the findings of McDermott and Pace (1960) if the effect of fertilisation is related/...

related to increased protein content.

The nutritional value of wheat flour proteins is limited by their marked deficiency in lysine and to a lesser extent tryptophan contents. This deficiency may be partly overcome by utilisation of the whole wheat proteins, particularly germ proteins, which have been shown to contain larger amounts of these amino acids. Moreover, heat treatment of flour proteins has been shown to result in some destruction of lysine and tryptophan to an extent of about 15% loss in lysine in baked bread (cf. Rosenberg and Rohdenburg, 1951). This has been attributed by Donehy and Pigman (1951) to the browning or Maillard reaction between sugars and lysine at high temperatures, resulting in its reduced availability.

2. Protein End Group Analysis

A certain amount of information has been obtained from end group analyses of gluten proteins. Mills (1955) reported a preliminary examination of the end groups present in his soluble DNP-gliadin fraction. Of the fifteen chromatographically distinct derivatives isolated after hydrolysis, only DNP-aspartic acid, DNP-serine and DNP-valine have been identified.

Wiseblatt et al. (1955) examined the peptides produced by the selective degradation of gluten proteins at the amino acid group of serine residues. Terminal group estimates indicated that each molecule contained several N-terminal serine residues, suggesting strong linking between peptide chains.

The end groups of gliadin from various wheats have been examined by Devenyl (1958). Differences in the amounts of phenylalanine residues were reported. Ramachandran and McConnell (1955) reported the presence of three N-terminal/...

N-terminal histidine residues in gliadin preparations. Tiunova (1961b), however, reported the presence of eight N-terminal amino acids in gliadin from two different wheats and couch grass. These included aspartic and glutamic acids, serine, threonine, alanine, valine, phenylalanine and leucine.

Glutens derived from nine different wheat varieties were shown by Winzor and Zentner (1962) to contain glutamic acid, aspartic acid, serine, threonine, glycine, alanine, valine and histidine in N-terminal positions. Leucine, phenylalanine and methionine were detected as end groups in some glutens. Quantitative differences were also reported.

3. Protein Fractionation

(a) Classical precipitation

Attempts to fractionate gluten proteins by relatively simple precipitation methods have been only moderately successful. Many early reports contain conflicting results which are difficult to interpret.

A large number of gluten fractions were isolated by Blish (1945) by sodium chloride precipitation from acetic acid solutions. Similarly McCalla and Rose (1935) fractionated gluten by magnesium sulphate precipitation from sodium salicylate dispersions. These fractions were shown to differ significantly in their physical and chemical properties, particularly in amino acid composition (cf. Stockelbach and Bailey, 1958). Blish (1945) however points out that the inadequacy of these methods may possibly be due to considerable molecular interaction resulting in the precipitation of mixtures of compounds in progressively varying proportions.

In/...

In a more recent paper, however, Tiunova (1960) claimed a sensitive and reproducible fractionation of gliadin from a common wheat and its parent forms, including couch grass, by the classical preparation procedure. Fractions were obtained from dilute NH_4OH solutions of gliadin by the addition of increasing amounts of ammonium sulphate. At least 6 fractions were observed by this method, with significant qualitative variations in several of the components. The component composition of gliadin soluble in varying concentrations of alcohol (30-85%) was also examined by this procedure (Tiunova, 1961b). On the basis of the extraction procedure and reproducibility, it was claimed that these fractions represented separate gliadin components (not molecular associations), although it was pointed out that some were probably heterogeneous mixtures with similar properties.

With the advent of newer more refined methods of fractionation, e.g. ultracentrifugation, chromatography and electrophoresis, methods based on classical precipitation procedures are no longer generally used. They are however useful for obtaining crude fractions of a particular species before further fractionation by more selective methods (cf. Quensel, 1942; Pence and Elder, 1953; Jones et al., 1959b; Dimler and Senti, 1959).

(b) Fractional solution

Many of the earliest attempts to fractionate gluten proteins were based on solubility methods, most of which have already been mentioned in another section (p. 10).

A more recent method was reported by Meredith et al. (1960a, 1960b).

A comparison of results obtained by the partition of gluten in a methanol¹-chloroform solvent/...

solvent, before and after dilute formic acid extraction suggested that the gluten complex is irreversibly split by acid treatment. The role of these fractions in the 3-dimensional gluten complex was proposed on the basis of their different physical and chemical characteristics.

A method for the fractionation of cadmium precipitated albumins of barley was reported by Rondelet and Lontie (1955). The cadmium-protein complexes were eluted from a Kieselguhr column at various pHs and ionic concentrations in a trimethylaminomethane buffer. At least four fractions were isolated in this way.

(c) Free boundary and paper electrophoresis

The techniques of free boundary and paper electrophoresis have recently been used with varying degrees of success for the fractionation of flour and gluten proteins. Much of the work has been confined to an investigation of the suitability of numerous buffer systems, although more recent reports now indicate that detailed fractionations may be achieved by these methods, especially by the moving boundary technique. Early attempts to fractionate wheat proteins using the free boundary electrophoresis method of Tiselius (1937) were hindered by the difficulty of obtaining adequately dispersed solutions which did not precipitate during the electrophoresis. By the use of unsatisfactory buffer systems, unsymmetrical patterns (with respect to ascending and descending limbs) were obtained, resulting in much conflicting information. These reports did however give some indication of the complexity of the gluten fractions.

In an early report Laws and France (1948) examined the acetic acid soluble proteins of flour by free boundary electrophoresis in a citric acid-disodium phosphate/...

phosphate buffer. No attempt was made to determine the component composition of the solutions, but merely to compare the patterns produced by flours of varying characteristics. The patterns, however, showed many abnormalities, attributed by Mills (1954) to convective disturbances, and no essential differences were observed in extracts from various flours.

Using this technique, in conjunction with a preliminary separation based on solubility methods, Mills (1954) reported the presence of at least four electrophoretically distinct fractions in gliadin. Again, the difficulty in obtaining symmetrical patterns was observed, thus hindering a proper interpretation of the results.

A significant advance in the electrophoretic separation of gluten proteins was reported by Jones et al. (1959b), who showed that symmetrical patterns could be obtained by the use of relatively low protein concentrations in conjunction with an aluminium lactate-lactic acid buffer (pH 3.1) of low ionic concentration. Under these conditions, gluten (previously washed in NaCl solution) was shown to contain at least six distinct components designated α_1 , α_2 , β_1 , β_2 , γ and ω in order of decreasing electrophoretic mobility, together with faster moving fractions taken to be albumins and globulins entrapped in the gluten ball.

Kelley and Koenig (1962, 1963) and Koenig (1963) using the free boundary technique in conjunction with a veronal buffer system, reported that sodium chloride extracts of flour contained at least 9 components. Acetic acid extracts of gluten (salt washed) in acid buffers (phosphate or aluminium lactate) were found to contain 3 main components, the proportion of which appeared to depend on the buffer pH. Acid extracts of flour were found to be similar to those from gluten, while alkaline extracts/...

extracts were significantly different. The presence of "salt soluble" proteins, thought to be trapped in the gluten, was again reported. Patterns obtained for gluten from defatted flours were similar, but significant differences were observed in gluten extracts of flours which had been previously exhaustively extracted with a series of lipid solvents (water saturated butanol and chloroform-methanol).

The use of filter paper electrophoresis was reported by Pence and Elder (1953) and Pence (1953) for the fraction of flour albumins. Using a sodium chloride-sodium cacodylate buffer system (pH 6), they observed at least 11 components, which were designated as $\alpha(1-4)$, $\beta(1-4)$ and $\gamma(1-3)$ according to the order of mobility and on similarity in behaviour at various pH values. Unsatisfactory patterns were however obtained by the moving boundary technique, although they did indicate the multiplicity of the fractions.

In a more recent paper, Zentner (1960) reported the fractionation of lactic acid extracts of untreated gluten by filter paper electrophoresis in a lactic acid buffer. Seven components were detected by this method, one of which could be stained with Oil Red O and was taken to be a lipoprotein.

(d) Chromatographic methods

The chromatographic separation of gluten proteins on carboxymethyl-cellulose (CMC) and diethylaminoethyl-cellulose (DEAE) columns has recently been reported as an effective method for the fractionation and isolation of protein components in preparative amounts.

Woychik et al. (1960) reported the chromatographic separation of gluten proteins in dilute acetic acid solution by stepwise elution with buffers of increasing hydrogen/...

hydrogen ion concentration. Preparative amounts of four fractions were obtained, three of which were successfully purified by rechromatography. The moving boundary electrophoresis technique of Jones et al. (1959b) was used to further investigate the composition and individuality of these fractions before and after rechromatography. Fractions isolated by this method have been successfully used to establish the relationship between separations obtained by moving boundary and starch gel electrophoresis (cf. Woychik et al., 1961b).

In a recent paper, Simmonds and Winzor (1961a, 1961b) reported the separation of the acetic acid soluble proteins of flour into eleven fractions by chromatography on CM-cellulose in an acetate buffer (pH 4.1). By incorporating a 1.0 M solution of dimethylformamide in elution buffers, it was possible to load the columns with larger amounts of protein (up to 100 mg.) allowing the isolation of larger fractions with increased resolution without the danger of precipitation.

This method was also used by Wrigley (1963) to examine the protein component composition of flour fractions isolated by air classification procedures. No differences were observed between the protein components of flour fractions and the parent flour.

Using a similar chromatographic technique, Coates and Simmonds (1961) and Simmonds (1963) reported the separation of eight pyrophosphate soluble proteins of flour on a DEAE-cellulose column.

(e) Gel electrophoresis

The method of electrophoresis in starch gel developed by Smithies (1955, 1959a, 1959b, 1962) for the fractionation of serum proteins has recently been shown to/...

to be a valuable tool for the separation of cereal and many other plant proteins.

Elton and Ewart (1960, 1962) first reported the application of this method to the fractionation of cereal proteins. Electrophoresis in gels incorporating the aluminium lactate buffer system of Jones et al. (1959b) permitted the side-by-side comparison of the individual components of various protein solutions. The gliadin, albumin and globulin fractions of wheat flour were examined by this method. Electrophoretic mobilities appeared to be in the order of globulins > albumins > gliadin, although much cross-contamination was reported which inhibited a proper classification. A maximum total of twenty components was observed. A comparison of the gliadin, albumin and globulin fractions for eight different wheat varieties showed that major differences existed only in the gliadin extracts. Aluminium lactate extracts of flour appeared to contain an almost complete spectrum of these components which differed markedly from similar extracts of barley, maize, oats and rye. Extracts of flour, previously washed in water saturated n-butanol to remove lipids, showed no significant differences in protein pattern.

Using the same conditions for electrophoresis, Kaminski (1962) examined the protein components of wheat flour removed by aluminium lactate solution and successive extractions in distilled water, 0.2 M NaCl solution, 70% ethanol and 0.5 N acetic acid solution. Considerable cross-contamination of fractions was observed and it was concluded that a classification of the soluble wheat proteins as either albumin or globulin cannot be made on the basis of solubility characteristics. Similar results were reported by Coulson and Sim (1961), and Coulson et al. (1962).

A similar electrophoretic separation of gluten proteins was carried out by Woychik/...

Woychik et al., (1961b). In this case the investigation was mainly confined to the slower moving acetic acid soluble components (gliadin), these being considered the true gluten fractions. Aluminium lactate buffers incorporating 3M urea were used in the gel preparation, as this was found to improve the definition of the bands, and allow the separation of larger amounts of protein. The gliadin fraction was resolved by this method into 8 components. It was also noted that an appreciable amount of protein failed to migrate into the gel. Elution of this material with subsequent electrophoresis by the moving boundary technique, showed that it corresponded to the fastest moving α_1 component. It was proposed that this corresponded to the major portion of the classical glutenin fraction. The presence of nine components was reported in a preliminary separation of water extracts of gluten.

In a recent paper, Jones and Dimler (1962) reported the separation of the protein components of air classified flour fractions by starch gel electrophoresis. Fractions of low and high protein contents contained identical slow moving components, but a larger proportion of the water soluble components were observed in the fractions of low protein content.

A method for the isolation of wheat protein fractions from starch gels during electrophoresis was recently reported by Elton and Ewart (1963). Fractions were eluted from a buffer-filled slot at intervals during the electrophoresis and their identity as single migrating species was established by re-electrophoresis.

(f) Ultracentrifuge and diffusion methods and molecular weight determinations

Because of the difficulty in obtaining suitable fractions of gluten proteins by conventional methods, there are few reports in the literature concerning the molecular/...

molecular size of these components.

McCalla and Gralen (1940, 1942) using the methods of sedimentation and diffusion reported an estimated minimal molecular weight of 35,000 for the most soluble fraction of gluten in sodium salicylate solution.

Using modern techniques for isolation of protein fractions and the method of ultracentrifugation, Jones et al. (1961) reported a weight average molecular weight of 2-3 million for glutenin. These preparations also contained smaller molecules of approximately 50,000. γ -gliadin was found to have a molecular weight of 47,000 while the 4 electrophoretically distinct β -gliadin components all appeared to be of approximately 42,000 molecular weight (cf. Jones et al. 1959b). These estimates were thought to be real and not a result of physical aggregation.

Nielson (cf. Pence and Mecham, 1962) recently reported that the glutenin fraction contains molecules ranging in molecular weight of 40,000 to 50,000 into the millions. Oxidation of this fraction by performic acid to split disulphide bonds resulted in the formation of a single sedimenting species with a molecular weight of about 21,000, suggesting that this may represent a basic monomeric unit of glutenin.

(g) Molecular sieve methods

Methods for the separation of proteins on the basis of molecular size have recently received a great deal of attention, for these not only provide a method of fractionating protein species, but also give some estimate of molecular sizes involved.

(i) Starch gel. One such method is the starch gel electrophoresis technique of Smithies (1955, 1959a, 1959b, 1962) which has been successfully adapted, as already described, for the separation of cereal proteins. In contrast to electrophoresis on paper/...

paper, the degree of migration of the protein species is dependent not only on the molecular charge but also on molecular size and shape.

In a recent paper, Smithies (1962) reported a study of the migration rates of various proteins of known molecular size in relation to the concentration of starch in the supporting medium. The rate of migration was found to be inversely proportional to the starch concentration, and the retardation coefficient, the fractional change in migration per unit change in the reciprocal starch concentration, was shown to be dependent only on the size of the ion and was independent of its charge. Preliminary results showed that in general, the magnitude of the retardation coefficient was related to the molecular size of the migrating protein and so could be used to determine the relative size of the components under investigation.

The possible development of this method to the determination of absolute molecular size was proposed.

(ii) Polyacrylamide gel. The success of the starch gel technique has stimulated interest in the use of other gels which possess similar molecular sieving properties.

The application of cross-linked polyacrylamide gels to the fractionation of serum proteins was first described by Davies and Ornstein (1959) and Raymond and Weintraub (1959). These gels have been found to be superior to starch in many ways, because of their inertness (cf. Cruft, 1962b) and ease of preparation and handling, while their transparency facilitates the scanning of patterns and quantitative estimation of the protein components. In contrast to starch, the degree of cross-linking may be controlled, allowing the preparation of gels appropriate to the molecular/...

molecular weight range of the component mixture.

This method has been applied by Evans et al. (1961) to the fractionation of peanut proteins, but as yet no published information is available on its application to cereal proteins, though Pence and Mecham (1962) have carried out preliminary studies.

The use of granulated polyacrylamide gels for the column chromatographic separation of protein mixtures was investigated by Hjerten (1962). The preparation of gels for the separation of substances over a wide range of molecular weights was reported (approximately two hundred to one million).

(iii) Sephadex. The application of particulate dextran gels to the desalting of proteins and other macromolecules originally described by Porath and Flodin (1959) has since been developed as an effective chromatographic method for the separation of water-soluble mixtures on the basis of molecular size (cf. Flodin and Porath, 1961). Specially prepared cross-linked dextran gels (Sephadex), are now commercially available for the separation of substances within the molecular weight ranges of less than 4000 up to above 200,000.

(iv) Dialysis. A further method for the estimation of molecular size of proteins was described by Craig et al. (1957) who found that the 50% escape times of a series of proteins through cellophane membranes fell roughly in the order of increasing molecular weights. Using a simple dialysis cell under controlled conditions, it was reported that molecular weights of up to 45,000 could be approximately estimated in this way.

(v) Electrodialysis. A modification of this method was later reported by Pierce and Free (1961) for the estimation of the molecular size of proteins during electrophoresis/.

electrophoresis in starch gel. This technique involved the implanting of cellophane membranes in the gel perpendicular to the direction of movement of the proteins. Chemical treatment with zinc chloride solutions allowed the preparation of a series of membranes of graded porosities suitable for the electro dialysis of proteins in the molecular weight range 10,000 to 100,000. Examination of a series of proteins of known molecular weight showed that for each particular species, there was a simple relationship between the degree of penetration (zero to maximum) and the membrane pore size, and this was closely related to the molecular weight of the protein.

EXPERIMENTAL

(A) MATERIALS

1. Commercial glutens and gluten fractions

Fielders gluten (Procea Products Ltd., London)
Colnbrook gluten (Procea Products Ltd., London)
Energen gluten (Energen Foods Co. Ltd., London)
BDH gluten (British Drug Houses Ltd., London)
Keever gluten (U.S. origin)
Midsol gluten (U.S. origin)
Pro-Vim gluten (U.S. origin)
Pro-80 gluten (U.S. origin)
Vicrum gluten (U.S. origin)
BDH gliadin (British Drug Houses Ltd., London)
Mann gliadin (Mann Research Labs. Inc., N.Y.)
BDH glutenin (British Drug Houses Ltd., London)

2. Gluten samples and fractions isolated by precipitation methods

Samples of gliadin and glutenin isolated from T. vulgare cv. Lutescens-329 and a perennial wheat M-2 (Lutescens-329 x Agropyrum glaucum) by precipitation methods, were kindly donated by Dr. N.A. Tiunova, Central Botanical Laboratory, Moscow.

3. Wedge and adhering protein

Samples of wedge and adhering protein were kindly donated by the late Professor Hess' laboratory (Technical University, Hannover).

4./...

4. Commercial flours

Bero (Plain flour, Thomas Bell and Son, Newcastle-upon-Tyne)

SCWS (Scottish Cooperative Wholesale Society, Junction Mills, Leith)

Air-classified flours (J. and R. Snodgrass Ltd., Washington Mills, Glasgow)

Air-classified flours (Spillers Ltd., Old Change House, Cannon Street,
London)

Strong, medium and weak flours (Spillers Ltd., Old Change House,
Cannon Street, London).

5. Commercial wheat germ samples

Bemax (Vitamins Ltd., London)

SCWS (Scottish Cooperative Wholesale Society, Junction Mills, Leith).

6. Fresh material

All fresh material used was obtained from wheat (Triticum vulgare, cv. Als) grown in the same experimental field (11 acres) (Seed Testing Station, Department of Agriculture for Scotland, East Craigs, Edinburgh).

7. Dormant seeds

Samples of ripe seeds were obtained from:

Mr. C. Rist and Dr. K.F. Finney, U.S.D.A., Peoria, Illinois, U.S.A.

(A. squarrosa, T. monococcum, dicoccum, durum, vulgare cv. Kaw, Chieftain x Tenmarq).

Dr. J.K. Jones, Department of Agricultural Botany, University of Reading.

(T. turgidum, polonicum, timopheevi, spelta, compactum).

Messrs W. Kay and Sons, Edinburgh (T. vulgare, cv. Champlain, Viking, Professeur Marchal, Capelle Desprez, Jufy).

Waite Agricultural Research Institute, University of Adelaide, Australia.

(T. vulgare cv. Gabo).

Bibby/...

Bibby and Sons Ltd., Liverpool (S. vulgare)

Seed Testing Station, Department of Agriculture for Scotland, East Craigs,
Edinburgh, (all other varieties of T. vulgare; H. vulgare cv. Ymer;
A. sativa cv. Blenda).

(a) Samples of Graminae other than wheat

Barley (<u>Hordeum vulgare</u> cv. Ymer)	Rice (<u>Oryza sativa</u>)
Oats (<u>Avena sativa</u> cv. Blenda)	Maize (<u>Zea mays</u>)
Rye (<u>Secale cereale</u>)	Milo (<u>Sorghum vulgare</u>)
Millet (<u>Panicum miliaceum</u>)	Couch grass (<u>Agropyrum repens</u>)

(b) Species of Triticum and closely related genera

Aegilops squarrosa (v. Typica No. 2)
Triticum monococcum L. (Cultivated einkorn) (CI, 2433)
T. dicoccum Schubl. (Emmer) (CI. 3686)
T. durum Desf. (cv. Mindum)
T. turgidum L. (cv. 1G1)
T. polonicum L. (cv. 7F1)
T. timopheevi Zhuk. (cv. 1T1)
T. vulgare Vill. (cv. Als)
T. spelta L. Spelt. (cv. unknown)
T. compactum Host. (cv. 8K2)

(c) Varieties of T. vulgare used in genetical studies

Jufy I	Welcome
Phoebus	Leda
Fylgia	Dominator
Jubiligen	Skandia

(d)/...

(d) General varieties of T. vulgare

Champlein	Vilmorin 27	Chieftain x Tenmarq
Viking	Maitre Pierre	Manitoba (cv. unknown)
Professeur Marchal	Minister	Russian (cv. unknown)
Capelle Desprez	Fylgia II	Squarehead's Master
Nord Desprez	Als	Squarehead's Master 3/14
N59	Gabo	Wilhelmina
Glasnevin Rosa	Kaw	Wilma

(B) METHODS

1. Laboratory isolation of gluten

Gluten was isolated from flour by the classical procedure of Beccari. Water, (65 ml.) was slowly added to the flour (100 g.) during mixing (Kenwood Chef, Domestic Food Mixer; speed 2) until a cohesive dough was formed. Mixing was continued for a further 3 minutes, after which the dough was allowed to stand for 30 minutes. Finally, the dough was transferred to a thin Polythene bag and washed free of starch in several portions (150 ml.) of distilled water. Starch was removed by gently squeezing the dough by hand until it was about to break up. At this point, the starch water was poured off, and fresh water added. This was continued until the wash water was free of starch. Yields of gluten depended to some extent on the type of flour used, but on average, 30-35% of crude gluten (10-12% dry weight) was obtained.

The hand washing procedure was found to be preferable to washing in a commercial gluten washer (H. Simon Ltd., Stockport) since the latter method gave low and variable yields of gluten, and was unsuitable for the isolation of gluten from pretreated, less cohesive doughs.

2. Dispersion of flour, dough and gluten

Samples were dispersed in the appropriate solvent for 30 minutes in a top drive macerator (Homogeniser No. 7700, MSE Ltd., London).

The mixture was centrifuged for 15 minutes at 3000 g., after which the supernatant was removed and centrifuged for a further 30 minutes at 25,000 g. (0°) in a refrigerated centrifuge (MSE Major, MSE Ltd., London).

Clarification/...

Clarification of solutions

Protein solutions normally contained a slight haze which could not be removed by centrifugation. In most cases, however, this did not affect the protein fractionation, but where necessary, it could be readily removed by filtration through a thick pad of paper pulp (J.B. Green Ltd., Tovil, Maidstone, Kent).

Concentration of protein solutions

Protein extracts were concentrated by dialysis against polyethylene glycol (Carbowax 20M, G.T. Gurr Ltd., London) contained in Visking dialysis tubing (Union Carbide International Co., NY; 18/32"), by partial or complete freeze-drying or by ultrafiltration where appropriate.

Preservation of protein solutions

Solutions were normally stored at -15° . Continual freezing and thawing, however, tended to cause significant protein precipitation, hence samples which were retained for long periods were freeze-dried before storage. Where possible, protein extracts were fractionated immediately after extraction, particularly for comparative studies.

3. Fractionation of protein components

(a) Paper electrophoresis

The vertical apparatus of Flynn and de Mayo (1951) and the horizontal apparatus of Wieland and Fischer (1948) (Messrs Shandon Ltd., London) were used for protein fractionation by paper electrophoresis. Separations were carried out on chromatography paper (Whatman No. 4) for 24 hours at 4 volts/...

4 volts cm^{-1} After electrophoresis, papers were dried at 100°C and stained as described below:

Protein stains:-

Nigrosine: (cf. Smithies, 1959a) Electrophoresis strips were stained in a solution of Nigrosine (0.5 mg./ml.) in glacial acetic acid/water/methanol (10:50:50) for 2 hours with repeated agitation, then washed in running water until a clear background was obtained. The papers were finally dried at 100°C . The use of acetic acid in the wash water was avoided as this tended to reduce the intensity of the patterns.

Naphthalene Black 12B (cf. Smithies, 1959a) and Azacarmin B (Pezold et al., 1959) were also used as protein stains but were found to be less effective than Nigrosine.

(b) Electrophoresis on cellulose acetate

Protein solutions were spotted onto a strip of cellulose acetate (20 cm. x 5 cm.; Oxoid, Oxo Ltd., London) and electrophoresis carried out using the horizontal apparatus of Wieland and Fischer (1948) as described in the previous section. Strips were dried and stained as for paper.

(c) Agar gel electrophoresis

Electrophoresis of protein solutions was carried out in aqueous agar gels (2%) following the method for starch gel electrophoresis (section (d)).

(d) Starch gel electrophoresis

(i) 8" x 4" gels

Initial experiments were carried out using starch gels 8" x 4" as shown/...

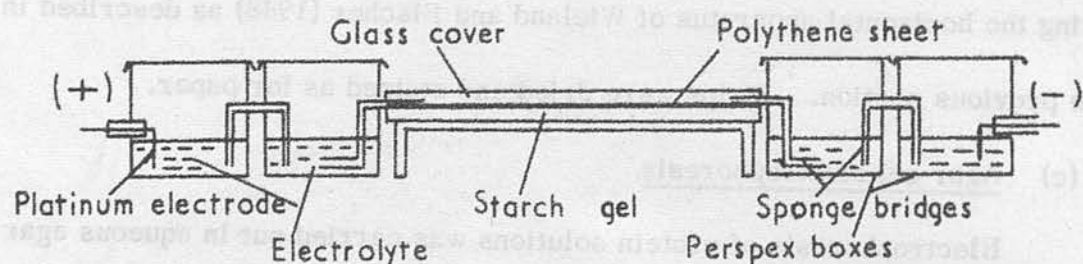
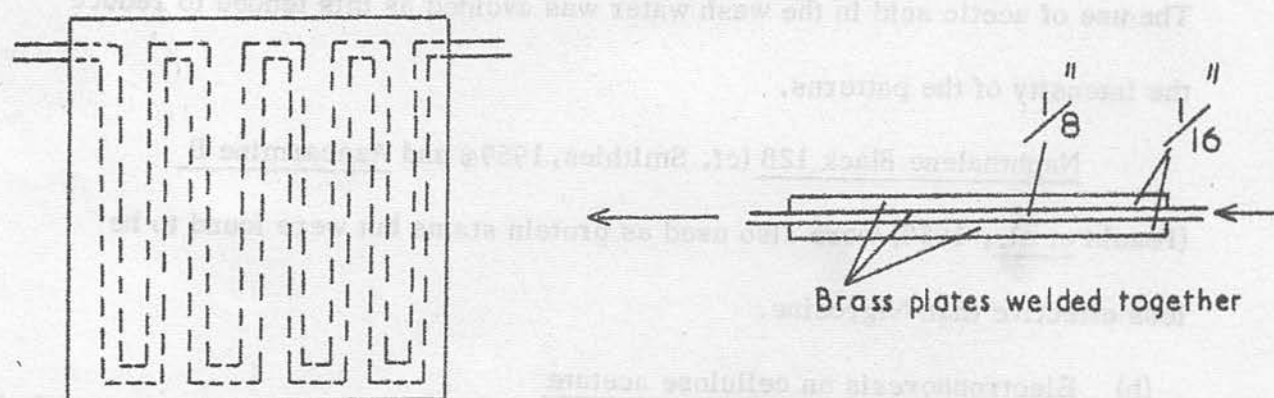
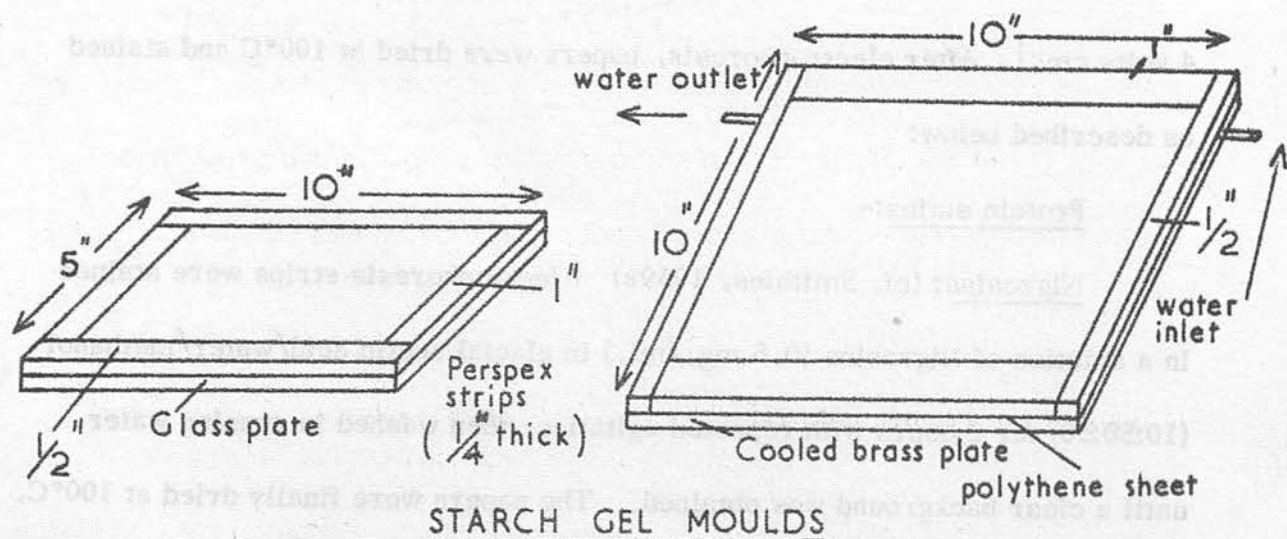


FIG. 1. STARCH GEL ELECTROPHORESIS APPARATUS

shown in Fig. 1. This allowed the separation of four samples per gel without cooling. A slight curvature of the patterns was observed due to localised heating towards the centre of the gel, but this did not hinder the interpretation of the results.

(ii) 9" x 8" gels

For comparative experiments it was found necessary to increase the size of the starch gels to 9" x 8". A gel mould incorporating a brass cooling plate insulated by a thick layer of polythene was constructed as shown in Fig. 1. Adequate cooling was obtained by allowing a constant stream of tap water to pass through the baffled base-plate. Up to nine samples could be accommodated in each gel.

(iii) Gel preparation (9" x 8") and electrophoresis

Starch powder (36.0 g., Connaught Starch-Hydrolysed, Connaught Medical Research Labs., Toronto, Canada) and urea (10 g.) were thoroughly mixed with the buffer solution (360 ml.) in a 500 ml. conical flask. The suspension was heated for 5-6 minutes over a bunsen flame, with constant agitation. After 2-3 minutes, the starch thickened and became gel-like, but on further heating, it became less viscous and was slowly allowed to boil. The flask was then transferred to a water pump and the the gel degassed for about 30 seconds, after which it was quickly poured into the mould. (The efficiency of the degassing procedure depended greatly on the buffer system employed. Small bubbles on the surface of the gel did not affect the electrophoretic separation.) The hot gel was then covered with a sheet of Polythene followed by a sheet of glass/...



glass, and any excess gel was gently squeezed from the mould. Finally, weights were placed on top of the cover and the gel allowed to cool at room temperature (3 hours). Cooling of the base-plate during this period tended to cause irregular setting of the gel. After cooling, slots ($\frac{1}{2}$ ") were cut in the gel $\frac{1}{2}$ " apart, care being taken to align them at the chosen position in the gel (depending on the buffer pH and direction of protein migration). A piece of filter paper (Whatman No. 3, $\frac{1}{2}$ " x $\frac{1}{4}$ ") was then soaked in the protein solution and after removing the excess solution the sample was gently slid into the gel slot. After positioning the samples, the electrode chambers were connected to the gel by means of cellulose sponge bridges and the Polythene and glass covers replaced. Electrophoresis was carried out at an external potential of 400 volts for 3 hours.

Direct current was supplied from a stabilised power pack capable of delivering 1500 volts DC and 200 mA. The external potential was measured using an Avometer connected between the platinum electrodes. The voltage gradient was measured by inserting platinum probes at either end of the gel.

(iv) Gel slicing

After electrophoresis, the Perspex sides of the gel mould ($\frac{1}{2}$ " x $\frac{1}{4}$ " x 10") were replaced by similar strips $\frac{1}{8}$ " thick. The gel was then sliced horizontally by a thin cheese wire (0.0068") conveniently held in a hack-saw frame. A sheet of Polythene was placed over the top half of the gel which was then gently peeled from the bottom half, while holding the gel mould in a vertical position. The top and bottom halves of the gel were then transferred to staining dishes by means of the supporting Polythene sheets.

(v)/...

(v) Gel staining

Nigrosine (cf. Smithies, 1959a). Gels were stained in a solution of

Nigrosine (0.5 mg./ml.) in glacial acetic acid/water/methanol (10:50:50, v/v/v) for at least 12 hours. Under these conditions, the patterns were immediately visible after removal of the staining solution. Washing was, however, continued for a further 5-6 hours in water to reduce the intensity of the background stain.

Naphthalene Black 12B (cf. Smithies, 1959a). Gels were stained in a

solution of Naphthalene Black 12B (0.5 mg./ml.) in glacial acetic acid/water/methanol (10:50:50, v/v/v) for approximately 12 hours. Under these conditions however, extended washing times, particularly with water, were required to reduce the intensity of the strongly stained background. Some visualisation of the patterns was possible after shorter periods of staining (5-6 hours), but some of the minor components were extremely weakly stained.

The use of glacial acetic acid, methanol or the usual protein precipitating agents (e.g. trichloroacetic acid, 10%) in the washing solution tended to reduce the intensity of the patterns.

(vi) Buffer-electrolyte solutions

The aluminium lactate-lactic acid buffer system of Jones et al. (1959b) described below, was used for all protein separations with the exception of leaf protein extracts, where a tris-citric acid buffer system was used.

(a) Buffer: Aluminium lactate (0.0176 M) - lactic acid (pH 3.2)

Electrolyte: Aluminium lactate (0.176 M) - lactic acid (pH 3.2)

(b)/...

(b) Buffer: Trishydroxymethylaminomethane (tris) (0.076 M) -
citric acid (0.005 M) (pH 8.65)

Electrolyte: Sodium hydroxide (0.1 M) - boric acid (0.3 M) (pH 8.81)

Separations were also attempted in sodium phosphate (0.01 M) and sodium hydroxide (0.0088 M) - boric acid (0.022 M) buffers, but these were considerably less effective.

(vii) Preparation of aluminium lactate-lactic acid buffers

Aluminium foil (5.47 g.) was first activated by immersion for about 30 seconds in a solution of mercuric chloride (1-2%) until bubbles appeared on the surface of the metal. After washing several times in distilled water, the aluminium was transferred to a beaker containing a solution of lactic acid (38.5 ml.) in approximately 400 ml. distilled water. The mixture was stirred overnight on a magnetic stirrer, after which it was filtered through a thick pad of Celite (Hyflo Supercel, L. Light and Co. Ltd.). The filtrate was then made up to 1 litre and the pH adjusted to 3.2 by addition of lactic acid. This stock solution was used as electrolyte. The buffer solution was prepared by diluting the stock solution 10 times and again adjusting the pH to 3.2 by further addition of lactic acid. Small amounts of toluene were added to stock solutions to prevent bacterial growth.

(viii) Addition of urea to buffers

The addition of urea (0.6 M) to aluminium lactate buffers before gel preparation was found to increase the rigidity of the gels and had a band-sharpening effect without any obvious alteration of the protein fractionation. Concentrations/...



FIG. 2. Automatic recording double beam reflectance densitometer for scanning electrophoresis strips and starch gels.

Concentrations of above 0.6 M however caused a precipitation of the protein components during the electrophoresis.

(ix) β -Amylase inhibition

β -Amylase activity in wheat protein extracts was found to cause significant degradation of the starch gel during electrophoresis, thus obscuring most of the slow-moving components (cf. Elton and Ewart, 1960, 1962). To inactivate the enzyme, solutions were placed in a boiling water bath for 2 minutes prior to electrophoretic separation (Jones et al., 1959b). Effective inhibition was also achieved by the addition of mercuric chloride solution (1%) to the protein extracts, but in some cases this resulted in a partial precipitation of protein material.

(x) Photography of starch gels

Illumination: Two 275 watt lamps (Photolita No. 1, Philips Ltd., London)

positioned above the gel.

Camera: M.P.P. Technical.

Film: FP3 (5" x 4") Ilford Ltd.

Exposure: 1/10 sec. at f8.

Developer: ID 11 (Ilford Ltd.) for 10 min.

(xi) Scanning of electrophoresis patterns

A commercial automatic recording double beam reflectance densitometer with integrator (Messrs Joyce, Loebel and Co. Ltd., Newcastle-upon-Tyne; "Chromoscan" 1961) was used for producing graphical scans (with peak integration values) of paper electrophoresis strips and starch gels (Fig. 2).

The sample carrier was slightly modified to allow the scanning of starch gel/...

gel strips. The elastic retainer behind the sample holder was found to be too tight and caused the gel to be squeezed through the front of the holder. This was replaced by a longer piece of elastic band ($8\frac{1}{4}" \times \frac{3}{4}"$). Starch gel strips were supported by a moist piece of thick filter paper (Whatman No. 3, $7\frac{1}{2}" \times 1"$) and placed on top of the plastic back-plate. The surface of the gel was then moistened and covered by a thin strip of cellulose acetate film ($8\frac{1}{4}" \times 1" \times 0.003"$).

The gel was then placed in the sample holder and clamped in position. Air bubbles were gently squeezed from beneath the film before scanning. Gels were scanned through a narrow slit ($\frac{1}{2}" \times 1/1000"$) using a light grey filter (0.5; Messrs Joyce, Loeb and Co. Ltd.). Patterns from the same gel were scanned with a constant base line setting.

The apparatus was used mainly to obtain a qualitative assessment of the electrophoresis patterns, but where required, a quantitative estimation of the protein fractions (relative) was obtained by integration of the recorded curves.

(e) Polyacrylamide gel electrophoresis

(i) Cyanogum 41 gelling agent

Cyanogum 41 Gelling Agent (American Cyanamid Co., N.Y., 10.0 g.) catalyst DMAPN (β -dimethylaminopropionitrile, 0.4 g.) and potassium ferricyanide (0.015 g.) were mixed with water (89.1 g.). Each was dissolved before the addition of the next component. Finally, ammonium persulphate (0.50 g.) was added with the minimum agitation. The solution was transferred to gel moulds ($8\frac{1}{2}" \times 2\frac{1}{2}" \times \frac{1}{4}"$) covered by a weighted sheet of Perspex and allowed to polymerise. Polymerisation took place in about 30 minutes to form a very tough/...

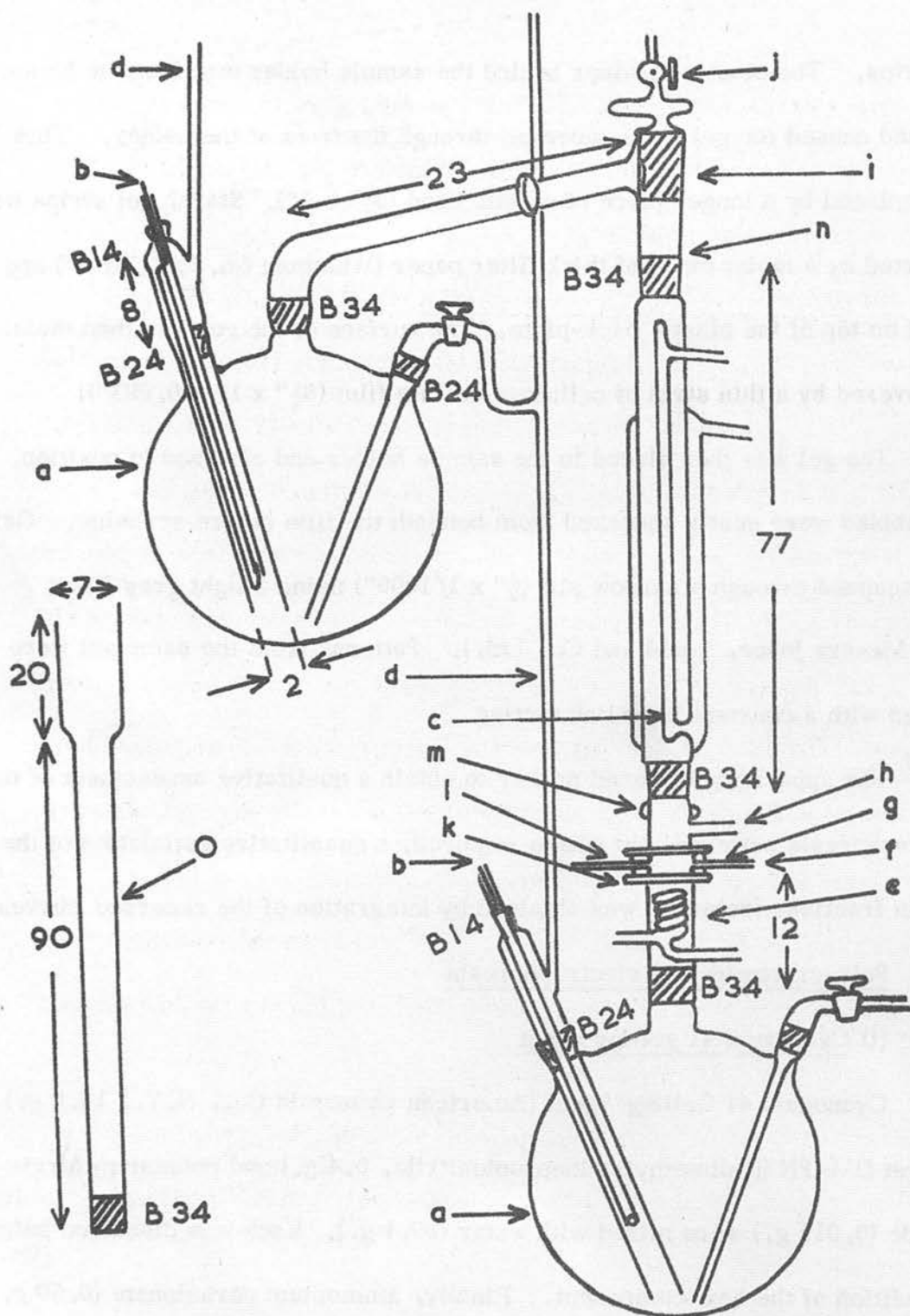


FIG. 3. Paper column electrophoresis apparatus. (a) Two litre electrode vessel, (b) platinum electrodes, (c) column, (d) gas outlet tube, (e) spiral cooling coil, (f) Perspex cooling ring (outlet not shown), (g) perforated Visking membrane, (h) sample outlet to fraction cutter, (i) electrode vessel isolation stopcock Q and Q SC 3/5, (j) eluent inlet tap, (k) Visking membrane, (l) buffer injection device for continuous elution, (m) paper column support, (o) reservoir for packing column inserted at (n). (all measurements in cms.)

tough, completely transparent, gel. Protein samples were inserted in the gel and electrophoresis carried out following the procedure for starch gel electrophoresis. No cooling was required.

(ii) Acrylamide-N,N'-methylenebisacrylamide (cf. Cruft, 1962b)

Acrylamide (2 g.) and N,N'-methylenebisacrylamide (40 mg.) were dissolved in the buffer solution (20 ml., aluminium lactate (0.017 M)-lactic acid, pH 3.2). Immediately before pouring, a 10% (v/v) solution (0.2 ml.) of N,N,N',N'-tetramethylethylenediamine in ethanol, followed by a freshly prepared 10% (w/v) aqueous solution (0.2 ml.) of ammonium persulphate were added with gentle stirring. The solution was then poured into a Perspex trough and covered by a weighted Perspex lid to expel air bubbles. After about 1 hour, the tough transparent gel was ready for use. Electrophoresis was carried out in a series of gels containing 10% to 7% acrylamide.

Notes: (i) It was essential to exclude air from the gel solutions, otherwise only an uneven layer of polymer was formed at the bottom of the gel mould, leaving a large volume of unreacted monomer on top.

(ii) Stock solutions of monomers and aluminium lactate tended to polymerise on standing; hence the two solutions were not mixed until immediately before gel preparation.

(f) Preparative column electrophoresis

(i) Cellulose column electrophoresis

The apparatus was assembled as shown in Fig. 3. The column was filled with buffer solution and then packed with cellulose powder (J.H. Munktell, Sweden)/...

Sweden). Even packing was ensured by employing a long extension tube containing a large reservoir at the top. A thick slurry of cellulose in the buffer solution was poured into the reservoir with constant stirring and the powder allowed to fall down the tube and settle in the column. After packing to the required height (65 cm.), the extension tube was removed and the sample to be fractionated was applied to the top of the column. The electrode chambers, containing buffer solution, were then connected to the column and electrophoresis carried out. The apparatus was so designed to allow either chromatographic elution of the material from the column after electrophoresis, or elution by continuous electrophoresis through a capillary take-off from the bottom of the column to a fraction collector (cf. Naughton and Taylor, 1960).

The method was tested by fractionation of a sample of "Universal indicator" (BDH, 0.5 ml.) in sodium tetraborate buffer solution (M/40). A potential of 800 volts was applied for 20 hours, giving clear resolution of the sample into 4 components.

A sample of a freeze-dried gluten/acetic acid extract (0.4 g.) was dissolved in acetic acid solution (N/100, 20 ml.) and applied to the column, previously filled with aluminium lactate (0.017 M)-lactic acid buffer (pH 3.2). Electrophoresis was carried out at 1000 volts with continuous take-off from the bottom of the column. 10 ml. fractions were collected on an automatic fraction collector (Locarte Co., London) for 180 hours. The optical density of each fraction was measured at 280 m μ (Hilger manual UV spectrophotometer).

(ii)/...

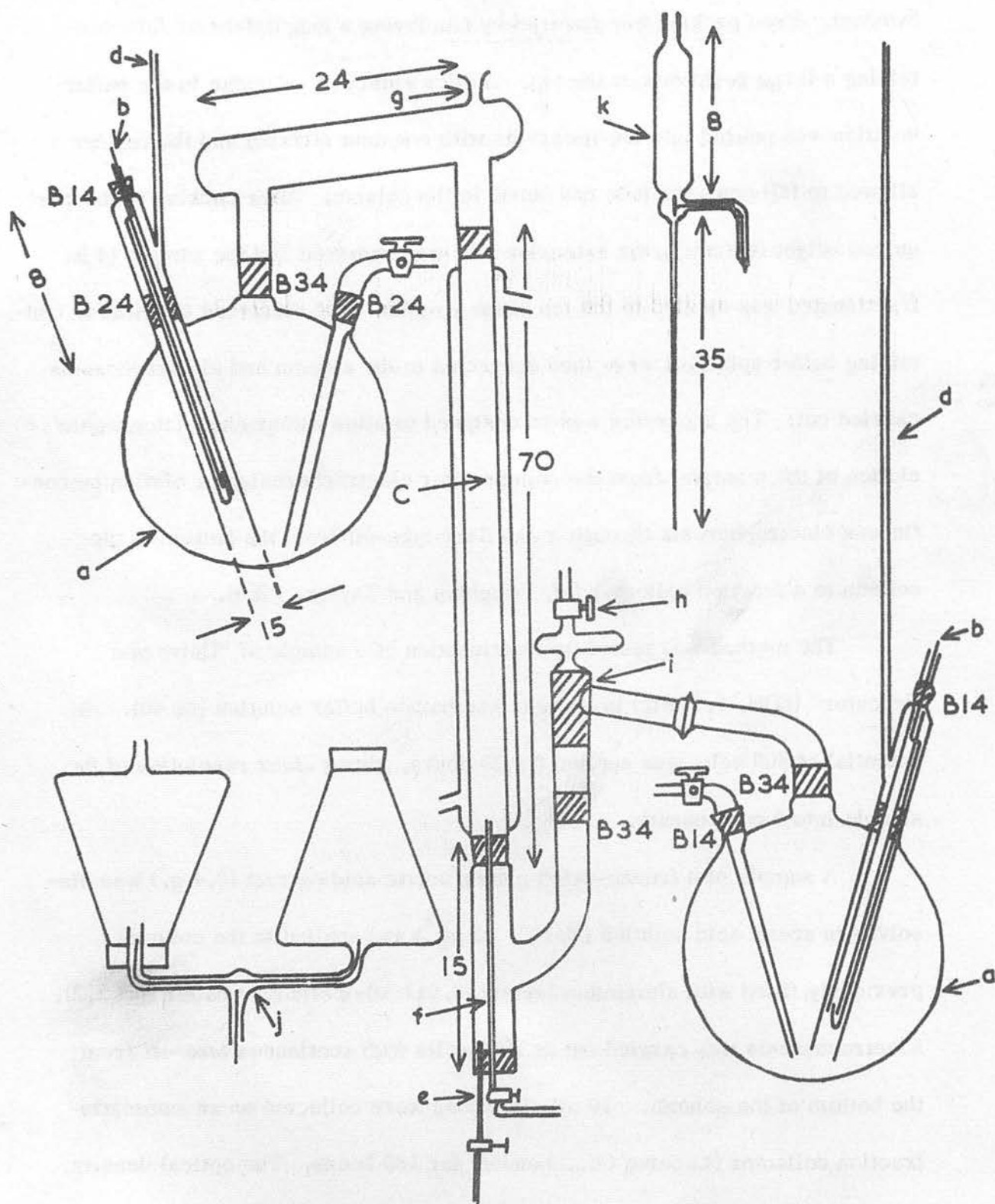


FIG. 4. Density gradient electrophoresis apparatus. (a) Two litre electrode vessel (b) platinum electrode, (c) column, (d) gas outlet tube, (e) drain tube, (f) density gradient inlet tube, (g) inlet for sample injection device (k), (h) eluent inlet stopcock, (i) electrode vessel isolation stopcock Q and Q SC 3/5, (j) device for establishing density gradient. (All measurements in cms.)

(ii) Density gradient electrophoresis (cf. Svensson, 1960)

The apparatus was assembled as shown in Fig. 4. The lower part of the column and electrode vessel were filled with "heavy" buffer (aluminium lactate (0.017 M)-lactic acid, pH 3.2, plus 500 g. sucrose per litre) and the upper part of the column and electrode vessel filled with "light" buffer (aluminium lactate-lactic acid, without sucrose) so that the interface between the two solutions was formed near the bottom of the column. The density gradient was laid by means of a mixing device consisting of two 250 ml. Erleinmayer flasks as shown. The buffer-sucrose solution entered the column at the interface between the light and heavy buffers. A decreasing density gradient of sucrose solution was thus laid from bottom to top of the column, the displaced light buffer being removed as necessary from the upper electrode vessel.

Attempts to inject the sample before laying the density gradient were unsuccessful since considerable mixing occurred during column preparation. The most suitable method of sample injection was obtained by replacing a small step from the top of the column with the sample solution, after adjusting to the same density.

The efficiency of the apparatus was tested by electrophoresis of a sample (0.1 g.) of an equal mixture of phenol red, metanil yellow, naphthol green and naphthylamine-azo-benzene p-sulphonic acid. The dyes were dissolved in a small sample of the buffer solution taken from the upper extremity of the density gradient. A 1 cm. layer was then slowly injected just below this level at such a point that it lay stationary in the gradient. A potential of 800 volts/...

volts was then applied for 36 hours, giving a clear resolution of the dye components.

A freeze-dried acetic acid extract of gluten (0.4 g.) was dissolved in dilute acetic acid solution (N/100, 20 ml.) containing a small amount (0.2 ml., 1%) of phenol red. A portion (10 ml.) of the buffer-sucrose solution was then removed from the top of the column. The density of the protein solution was adjusted to that of the buffer solution and a 2 cm. layer was then injected into the column. A potential of 1000 volts was applied for 48 hours. The protein material migrated down the column, while the phenol red migrated in the opposite direction, dispersing in the upper electrode vessel. Fractions were eluted from the top of the column by passing a slow current of heavy buffer through tap H (Fig. 4). The absorption of the eluent was measured at 254 m μ in a recording UV spectrophotometer (Uvicord 4701A, LKB-Produkter, Sweden) before collection (2 ml. samples) in an automatic fraction collector, or at 280 m μ as in section (i).

Similar fractionations were carried out over 14, 24, 64 and 68 hours.

(g) Molecular sieve methods

(i) Electrophoresis markers for wheat proteins

The electrophoretic mobility of wheat proteins in starch gel was compared with that of a number of substances which were readily available and could be fractionated at a low pH. A side-by-side comparison of the protein components of the following substances was carried out as described in section (d), p. 50.

Papain/...

Papain (BDH Ltd.)	}	0.2 g. in acetic acid solution (N/100, 10 ml.)
Pepsin (BDH Ltd.)		
Bovine glycoprotein (Fraction VI, L. Light and Co. Ltd.)		
β -Amylase (ex-barley, L. Light and Co. Ltd.)		
Egg white (filtered)		
Gluten (Energen, 5 g.) in acetic acid solution (N/100, 50 ml.)		

(ii) Estimation of molecular size by membrane filtration (cf. Craig
et al., 1957; Pierce and Free, 1961).

a Untreated Visking membranes

Electrodialysis experiments were carried out following the method of Pierce and Free (1961). A series of untreated cellophane membranes (Visking, Union Carbide International Co., NY.; $\frac{3}{4}$ " x $\frac{1}{4}$ "") were prepared from dialysis tubing of inflated diameters 8/32", 18/32", 20/32", 36/32" and 80/32", and inserted in starch gels at a distance of 1 cm. from the point of sample insertion, at right angles to the direction of protein migration.

Samples of an aqueous flour extract (Als) were then fractionated under normal electrophoretic conditions with aqueous solutions of bovine serum albumin (L. Light and Co. Ltd.), bovine haemoglobin (Armour Pharmaceutical Co. Ltd.), ovalbumin (L. Light and Co. Ltd.) and chymotrypsin (L. Light and Co. Ltd.) as controls.

b Zinc chloride treated membranes

A series of Visking membranes ($\frac{3}{4}$ " x $\frac{1}{4}$ "", 20/32" tubing) of varying porosities were prepared following the procedure of Pierce and Free (1961).
Membranes/...

Membranes were immersed in zinc chloride solutions (52% to 60%) for 20 minutes at 25°, followed by alternate 5 minute rinses in distilled water, 7.5% HCl and distilled water and finally stored in an aqueous ethanol solution (25%).

Membranes, classified according to their zinc chloride treatment, were then used for the filtration of flour proteins and control samples during electrophoresis in starch gel as described in the previous section.

c Millipore filters (cf. Fuchs and Gorin, 1961)

Experiments described in section a were repeated using a series of membranes (Millipore Filter Corporation, Bedford, Mass., USA) of pore sizes 5 μ , 1.2 μ , 100 m μ , 50 m μ and 10 m μ . For each membrane, an aqueous flour extract was fractionated with the series of controls listed above.

Collected results from the above are shown in Fig. 9.

(iii) Sephadex filtration

The following grades of Sephadex (Pharmacia, Sweden) were investigated for the filtration and fractionation of wheat proteins.

G.25 - absorbing molecules of molecular weight less than 4,000

G.50 - absorbing molecules of molecular weight less than 10,000

G.75 - absorbing molecules of molecular weight less than 50,000

G.100 - absorbing molecules of molecular weight less than 100,000

G.200 - absorbing molecules of molecular weight less than 200,000

Samples used in these experiments were of the coarse or bread form (G100) since adequate flow rates were not obtained using finer grades. Gel particles were allowed to soak in distilled water for at least 24 hours (48 hours for/...

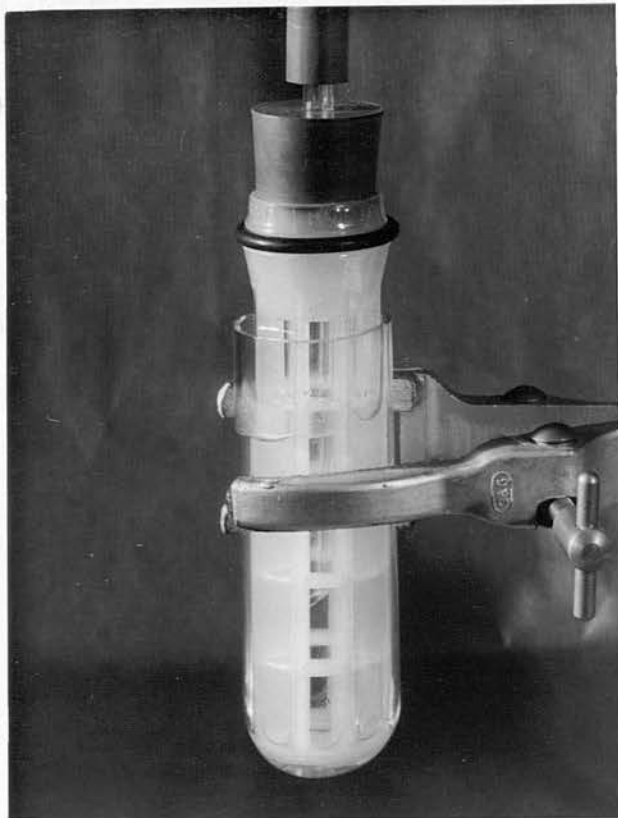


FIG. 5. LKB-Produkter Ultrafilter

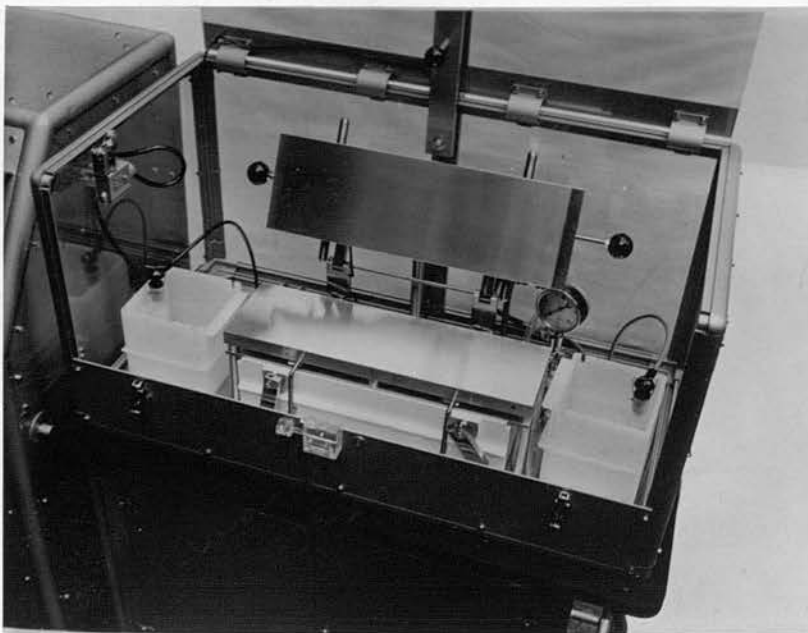


FIG. 6. High Voltage Electrophoresis Apparatus.

for G.100 and G.200) before packing in columns. The supernatant was decanted from the gel at intervals and replaced with fresh distilled water until free from fine particles. The gel was then packed in a chromatographic column (1.5 cm. x 25 cm.) fitted with a sintered glass disc (porosity 4). An extension column (1.5 cm. x 25 cm.) was used to ensure even packing. A filter paper disc was placed on top of the column to prevent disturbance during sample application. Before use, the gel was washed with 2 x 100 ml. portions of distilled water.

A 2 cm. layer (2 ml.) of an aqueous flour extract was applied to the column by lowering the level of water above the gel until the top of the filter paper disc was just visible. The sample was then slowly applied by syringe and allowed to pass into the gel. 2 x 2 ml. portions of distilled water were then added in a similar fashion before finally connecting the column to a water reservoir. The flow rate was adjusted to 15 ml. per hour. The eluent was passed by way of fine nylon capillary tubing through a recording UV spectrophotometer (Uvicord, LKB-Produkter, Sweden) and the absorption measured at 254 m μ and recorded automatically (Ether Microsen recorder, Ether Ltd., Stevenage, Herts.). Similar fractionations were carried out in larger columns (3 cm. x 30 cm.; 5 cm. x 30 cm.). Fractions shown in Fig. 11 were later examined by starch gel electrophoresis.

4. Fractionation of protein subcomponents

Ultrafiltration. Free amino acids and peptides were removed from protein solutions by ultrafiltration through evacuated dialysis tubing (Visking, 18/32") supported on a slotted nylon frame (LKB-Produkter, Sweden) as shown in/...

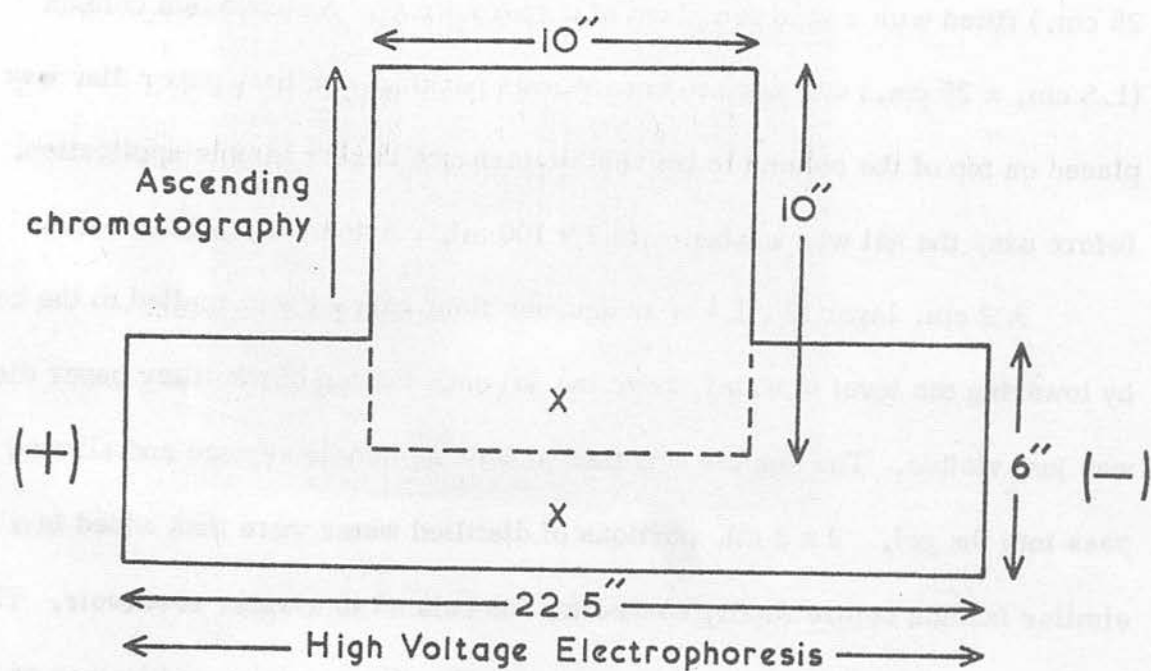


FIG. 7. Preparation of paper for electrochromatography

in Fig. 5. Porous polyethylene tubing (cf. Siegelman and Firer, 1962) was also found to be an effective supporting medium, but was less suitable for small volumes of solution.

Paper chromatography. (i) Two-dimensional ascending paper chromatography was carried out in a modified Datta frame system (Datta et al., 1950; Smith, 1958; Messrs Aimer Ltd., London) allowing five papers (Whatman No. 1, 10" x 10") to be run concurrently.

(ii) For more detailed separations, a combination of descending and ascending techniques were used. Separations were carried out on Whatman No. 1 chromatography paper (16" x 9") using the descending techniques along the long axis. For ascending runs, papers were rolled in a cylindrical shape and allowed to stand in a tray of solvent placed in the base of the chromatography tank.

Electrochromatography. Two-dimensional separation of protein partial hydrolysates was carried out first by high voltage electrophoresis followed by ascending chromatography in the direction at right angles to the electrophoretic separations. Papers (Whatman No. 3) were prepared as shown in Fig. 7, allowing the electrophoresis and staining of a control sample before further chromatographic separation.

High voltage electrophoresis. The apparatus (Fig. 6) was essentially that of Gross (1959, 1961) in commercial form (Messrs Locarte Co., London). The paper was cooled by two stainless steel heat exchangers, with tap water as coolant, and insulated by polyethylene film (1 mm. thickness). A pneumatic pressure device ensured evenness of pressure and cooling. A power supply, with/...

with maximum output of 10 K volts at 100 mA was incorporated in the apparatus

Chromatography. After electrophoretic separation, the paper was cut as shown in Fig. 7 and ascending chromatography carried out in the Datta frame system as already described.

5. Nitrogen determinations

Nitrogen determinations were carried out using the following micro-Kjeldahl method.

50% Sulphuric acid (2 ml.) and catalyst (0.15 g.) - a mixture of potassium sulphate (150 g., A.R.), selenium, (5 g., A.R.) and mercuric oxide (10 g., A.R.) were added to the protein sample and the mixture heated on a digestion stand until clearing of the solution had occurred (approximately 24 hours).

The contents of the digestion tube were then transferred to a Markham micro-Kjeldahl steam distillation apparatus (cf. King and Wootton, 1959) and a solution of 40% sodium hydroxide (10 ml.) was added. The liberated ammonia was absorbed in a boric acid solution (20 g./l., A.R.) containing a mixed indicator (equal volumes of 0.033% ethanolic bromocresol green and 0.066% methyl red). The distillation was continued for 3 minutes after the first appearance of ammonia. The solution was finally titrated with N/70 sulphuric acid solution.

6. Moisture determinations

Moisture contents of finely ground samples were determined by drying at 105°C for 24 hours.

(C)/...

(C) PROTEIN STUDIES

1. Solubility of wheat proteins

(a) Initial experiments by paper electrophoresis

Wet crude gluten (10 g., ex-Bero flour) was dispersed in the following solvents (50 ml.) as already described (p. 48):

- (i) Lactic acid (N/100)
- (ii) Lactic acid (N/100) - EDTA (0.1%)
- (iii) Lactic acid (N/100) - acetyl acetone (0.1%)
- (iv) Acetic acid (N/100)
- (v) Acetic acid (N/100) - EDTA (0.1%)
- (vi) Acetic acid (N/100) - acetyl acetone (0.1%)
- (vii) Formic acid (N/100)
- (viii) Sodium salicylate solution (10%)
- (ix) Sodium tetraborate solution (M/40)
- (x) Sodium hydroxide solution (N/10)

Samples of each solution were examined by paper electrophoresis using the following electrolytes:

Lactic acid (N/100) - solutions (i) to (iii)

Acetic acid (N/100) - solutions (iv) to (viii)

Sodium tetraborate solution (M/40) - solutions (ix) and (x)

Stain: Nigrosine (p. 50).

(b) By starch gel electrophoresis

(i) Samples of flour (30 g., Bero) and freshly extracted crude gluten (10 g., ex-Bero flour) were dispersed in the following solvents:

Distilled water/...

Distilled water (pH 5.6)

Sodium chloride solution (2M, M, 0.5 M, 0.2 M, 0.1 M, and 0.02 M)

Aqueous ethanol (70%)

Dilute acetic acid (N/100)

Portions of each solution (0.2 ml.) were examined by starch gel electrophoresis.

(ii) Solubility in sodium pyrophosphate solution (Simmonds and Winzor, 1961).

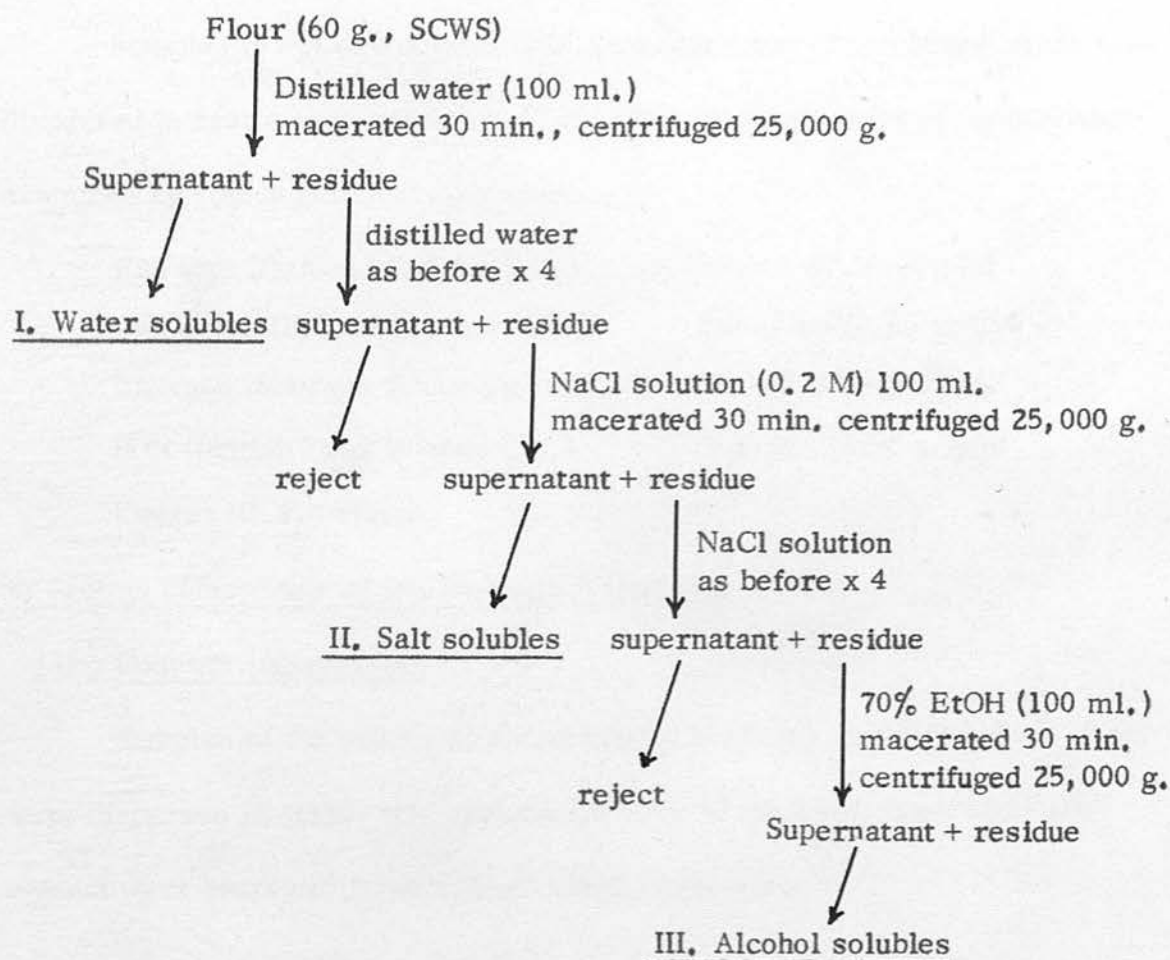
The solubility of flour proteins in sodium pyrophosphate solution was investigated following the extraction procedure of Simmonds and Winzor (1961).

A sample of flour (100 g., Bero) was dispersed in water-saturated n-butanol (400 ml.) for 30 minutes. The suspension was centrifuged at 3000 g. for 30 minutes and the supernatant rejected. 0.01 M sodium pyrophosphate solution (150 ml., pH 7.0) was then added to the residue and the mixture macerated for 30 minutes. After centrifuging for 15 minutes at 3000 g., the supernatant was removed and centrifuged for a further 30 minutes at 25,000 g. The extraction procedure was repeated with a further 4 x 150 ml. portions of pyrophosphate solution and samples of each extract were retained for electrophoresis. Finally, the residue from each extraction was combined, macerated for 30 minutes in acetic acid solution (N/100, 150 ml.) and centrifuged as before.

Samples of each solution were examined by starch gel electrophoresis as described in the previous section.

(iii) Group separation of flour proteins. A group separation of the protein components of flour was carried out in an attempt to classify them as far as possible on the basis of solubility, following the classical procedures. Samples/...

Samples of each extract were examined by starch gel electrophoresis.



2. Effect of dielectric constant on solubility

Extracts of freshly isolated crude gluten (10 g., ex-Bero flour) in the following solvents (50 ml.) were examined by starch gel electrophoresis. Dielectric constants are given in each case (cf. Ackrell and Couper, 1961).

- (a) 2M N-methylformamide (182.4)
- (b) 2M Formamide (109.5)
- (c) 2M N-N -dimethylformamide (36.7)
- (d) 2M Urea
- (e) Distilled water (80).

3. Protein components of commercial gluten samples

Samples (5 g.) of commercially available dried gluten listed below were dispersed in acetic acid solution (N/100, 50 ml.) and portions of each extract examined by starch gel electrophoresis.

Fielders (Procea Products Ltd.)	Midsol (U.S. origin)
Colnbrook (Procea Products Ltd.)	Pro-Vim (U.S. origin)
Energen (Energen Foods Co. Ltd.)	Pro-80 (U.S. origin)
BDH (British Drug Houses Ltd.)	Vicrum (U.S. origin)
Keever (U.S. origin).	

4. Protein components of isolated gluten fractions

(a) Commercial samples

Samples of the following commercially available gluten fractions (5 g.) were dispersed in acetic acid solution (N/100, 50 ml.) and portions of each extract were examined by starch gel electrophoresis.

(i) Gliadin (BDH Ltd.)

(ii) Gliadin (Mann Research Labs. Inc., N. Y.)

(iii) Glutenin (BDH Ltd.)

(b) Precipitated fractions

Gliadin and glutenin fractions were prepared by Tiunova (1960, 1961_b) from flour samples of T. vulgare cv. Lutescens-329 and a perennial wheat M-2 (Lutescens-329 x Agropyrum glaucum) as follows:

Gliadin fractions were prepared by extraction of defatted flour in 40, 50, 60, 70 and 80% (v/v) ethanol, precipitated by addition to distilled acetone and dried in vacuo over H₂SO₄.

Glutenin/...

Glutenin fractions were prepared from sodium hydroxide extracts (0.2%) of flour after sodium chloride (1 M) and ethanol (as above) extraction.

Samples (0.5 g.) of each fraction were dispersed in acetic acid solution (N/100, 6 ml.) and portions of the extracts examined by starch gel electrophoresis.

5. Physical treatment of wheat proteins

(a) Heat stability

Effect of heat on isolated gluten

Samples of freshly isolated crude gluten (10 g., ex-Bero flour) were heated for periods of 30 minutes, 60 minutes and until dry at 100°C. Each sample was then dispersed in acetic acid solution (N/100, 50 ml.) and portions of the extracts were fractionated by starch gel electrophoresis.

The experiment was repeated using commercially dried samples of gluten (5 g., Vicrum).

Effect of heat on gluten solutions

(i) Gluten dispersions. Freshly isolated crude gluten (10 g., ex-Bero flour) was dispersed in acetic acid solution (N/100, 50 ml.) for 15 minutes. The dispersion was then heated for 15 minutes on a boiling water bath, and then dispersed for a further 15 minutes.

(ii) Gluten extracts. Acetic acid extracts (10 ml., N/100) of freshly isolated crude gluten (ex-Bero flour) were heated for periods of 2, 10, 15 and 30 minutes in a boiling water bath. Where necessary, the solutions were centrifuged/...

centrifuged at 3,000 g. for 15 minutes.

Samples of solutions from (i) and (ii) with an untreated gluten/acetic acid extract, were examined by starch gel electrophoresis.

(b) Ultrasonic treatment of gluten proteins

A freshly isolated sample of crude gluten (10 g., ex-SCWS flour) was macerated for 5 minutes in acetic acid solution (N/100, 50 ml.). The dispersion was then transferred to an ultrasonic generator and subjected to ultrasonic vibration (20 W/cm^2 , MSE Ultrasonic Disintegrator No. 3000) for 30 minutes. A further sample was similarly treated for 60 minutes. After treatment, the dispersions were centrifuged for 30 minutes at 3,000 g. and portions of each extract, with an untreated sample, were fractionated by starch gel electrophoresis.

(c) γ -irradiation of gluten, flour and wheat seeds

Samples of commercially dried gluten (Vicrum), flour (SCWS), and wheat seeds (Jufy) were irradiated with γ -rays (8 K rad, Co^{60} source) over a period of 4 days. The wheat seeds were milled after treatment and acetic acid extracts of each sample, with untreated controls, were examined by starch gel electrophoresis.

The experiment was repeated at a higher level of irradiation (32.5×10^6 rad).

(d) Mechanical effects on gluten

Mastication. Freshly isolated gluten samples (10 g., ex-SCWS flour) were masticated (MSE Micromasticator No. 77313) for periods of 10, 20, 30 and/...

and 60 minutes at constant temperature. Samples were then dispersed in acetic acid solution (N/100, 50 ml.) in the usual manner and portions of each extract fractionated by starch gel electrophoresis.

(e) Effect of dough ageing on protein solubility

A series of doughs were prepared (25 g. flour, SCWS; 15 ml. distilled water) and aged for periods of 5 minutes, 30 minutes and 2 hours. Each sample was then dispersed in distilled water (30 ml., 30 min.) and portions of the extracts retained for electrophoresis. Gluten from a fourth dough, aged for 2 hours, was isolated in small amounts of distilled water to a total of 85 ml. The starch slurry was centrifuged and the supernatant retained. The isolated gluten was dispersed in distilled water (30 ml., 30 min.) and a portion of the extract retained for electrophoresis.

Samples of each extract, with an untreated aqueous flour extract as control, were finally examined by starch gel electrophoresis.

(f) Ageing of protein solutions

Acetic acid extracts of freshly isolated crude gluten (10 g., ex-SCWS flour) were allowed to stand for 24 hours, 48 hours, 72 hours, 7 days and 14 days at +4°C. Solutions were then allowed to reach room temperature, centrifuged where necessary and fractionated by starch gel electrophoresis. Control samples of freshly extracted material were examined with each aged solution.

6./...

6. Chemical treatment of wheat proteins

(a) Lipid removal

(i) Exhaustive extraction (cf. Mecham and Mohammad, 1955;

Jones *et al.*, 1959b.

A sample of flour (100 g., SCWS) was macerated for 30 minutes in n-butanol (250 ml.). After allowing the flour to settle, the solvent was removed by decantation and the dispersion repeated with a further portion of n-butanol (250 ml.). The isolated flour was then allowed to stand overnight in n-butanol (500 ml.), washed in a further 250 ml. of solvent, centrifuged and allowed to dry in air. Sufficient water was then added to form a coherent mass and the "dough" allowed to age for 30 minutes. Subsequent attempts to isolate the gluten were however unsuccessful, since the material dispersed immediately on addition of water. A portion of the dough (40 g.) was dispersed in acetic acid solution (N/100, 35 ml.) and a sample of the extract examined by starch gel electrophoresis.

(ii) Partial lipid removal

A sample of flour (100 g., SCWS) was dispersed in n-butanol (250 ml.) for 30 minutes. The solvent was removed by decantation and the dispersion repeated using a further 2 x 250 ml. portion of n-butanol. The flour was then allowed to dry in air, and mixed with sufficient water to form a dough. After ageing for 30 minutes, the gluten was isolated and dispersed in acetic acid solution (N/100, 50 ml.). A portion of the extract was finally examined by starch gel electrophoresis.

(b)/...

(b) Effect of redox systems on wheat proteins

(i) Incorporation during dough preparation

A sample of flour (100 g., SCWS) was mixed to a dough with distilled water (60 ml.) containing a small amount of potassium bromate (60 p.p.m.). After ageing for 30 minutes, the gluten was isolated and a portion of the gluten (10 g.) was extracted in distilled water (50 ml.).

The procedure was repeated incorporating potassium iodate (60 p.p.m.), cysteine (0.01 M) and reduced glutathione (0.01 M) during dough preparation.

Samples of each solution with an untreated extract were examined by starch gel electrophoresis.

(ii) Flour incubation

A sample of flour (5 g., SCWS) was suspended in distilled water (10 ml.) containing a small amount of potassium bromate (60 p.p.m.). The mixture was allowed to stand for 72 hours with continuous agitation. Samples of the suspension (2 ml.) were withdrawn after 24 hours, 48 hours and 72 hours and centrifuged.

Samples containing potassium iodate (60 p.p.m.), cysteine (0.01 M) and reduced glutathione (0.01 M) were similarly prepared, together with a control sample containing only an aqueous flour suspension.

Portions of each extract were finally examined by starch gel electrophoresis.

7./...

7. Biochemical treatment of wheat proteins

(a) Proteolytic enzymes

(i) Partial hydrolysis

Solutions of trypsin, papain and pepsin were prepared as follows:

Trypsin (1.5 mg.) in N/100 acetic acid solution (10 ml.)

Papain (1.5 mg.) + cysteine (1.58 mg.) in N/100 acetic acid solution (10 ml.)

Pepsin (1.5 mg.) in N/100 acetic acid solution (10 ml.)

Portions (6 ml.) of each enzyme solution were added to freshly prepared gluten/acetic acid extracts (10 ml.). Control samples, containing cysteine and gluten only were similarly prepared. Solutions were then incubated at 32° for 72 hours with continuous agitation. Samples (2 ml.) were withdrawn after 24 hours, 48 hours and 72 hours and examined by starch gel electrophoresis.

(ii) Protein "fingerprinting" (cf. Ingram, 1958)

Enzyme hydrolysis. Gluten/acetic acid extracts were prepared as already described. To each solution (10 ml.) was added 1 ml. of a papain/cysteine solution prepared as in the previous section. The samples were then incubated for 24 hours at 32° with constant agitation. Portions of each solution (2 ml.) were withdrawn after 2 hours, 8 hours and 24 hours and ultrafiltered.

Electrochromatographic separation of the ultrafiltrates from the above was carried out as already described (p. 64), under the following conditions:

Electrophoresis/...

Electrophoresis: Electrolyte: (i) pyridine/acetic acid/water (10:0.4:89.6)

(ii) acetic acid solution (N/10)

6 K volts at 60 mA: 20 min.

Chromatography: Solvent: n-butanol/acetic acid/water (12:3:5) for 7 hours

Stain: (i) Ninhydrin in acetone (0.2%)

(ii) tert-butyl-hypochlorite; KI/starch

(Mazur, et al., 1962).

(b) Phospholipases and Amylases

Samples of crude phospholipase D (0.6%, ex-cabbage leaf) and fungal α -amylase (0.6%, Novadel Ltd.) were incorporated in separate glutes during dough preparation as previously described (p. 74). After incubation for 48 hours at 30°C, the isolated glutes were dispersed in acetic acid solution (N/100, 50 ml.) and portions of each extract examined by starch gel electrophoresis.

8. Biological techniques

(a) Protein composition of the mature wheat seed

(i) Anatomical separation

Ripe wheat samples (Als) obtained from field plots were separated as far as possible by hand dissection into various anatomical parts. The protein components of these sections were extracted and examined, mainly under the conditions developed for the fractionation of endosperm proteins and associated protein subcomponents.

Pericarp/...

Pericarp. Husks were removed from ripe wheat spikes (Als) by hand threshing, and ground to a fine powder in liquid air. A sample (5 g.) was dispersed in acetic acid solution (N/100, 30 ml.) following the procedure for gluten extraction. Finally, the extract was concentrated by ultrafiltration to 1/3 of the original volume and portions of the ultrafiltrate and residual solution examined by 2-dimensional chromatography and starch gel electrophoresis in an aluminium lactate/lactic acid buffer.

Chromatographic solvents: (i) n-butanol/glacial acetic acid/water
(12:3:5; 7 hours)

(ii) phenol/ammonia (500 g. in 125 ml. water/
1ml. 0.88 NH₄OH; 8 hours)

Stain: Ninhydrin (0.2%) in acetone.

Testa and aleurone layer. Ripe seeds (Als, approx. 100) were milled by hand and the flour removed by sieving (100 mesh). The residue containing mainly testa + aleurone cells was washed several times in distilled water to remove the bulk of the adhering endosperm material, and finally ground to a fine powder in liquid air.

A sample (2 g.) of the material was dispersed in acetic acid solution (N/100, 10 ml.), and the extract concentrated as before by ultrafiltration.

Portions of the ultrafiltrate and residual solution were examined as described in the previous section.

Embryo. Embryos (including scutellum) were removed from ripe seeds/...

seeds (Als, approx. 100) by hand dissection and milled to a fine powder in liquid air. A portion of the material (0.1 g.) was dispersed in acetic acid solution (N/100, 5 ml.) and the extract concentrated by ultrafiltration as in the previous section. Samples of the ultrafiltrate and residual solution were examined by chromatography and starch gel electrophoresis as before.

Extracts of two commercially available samples of wheat germ were similarly examined (Bemax, Vitamins Ltd., London; Isolated wheat germ, SCWS).

Endosperm. Ripe seeds (Als) were milled by hand and the endosperm material removed by sieving (100 mesh). A sample of the flour (2 g.) was dispersed in acetic acid solution (N/100, 6 ml.) and the extract ultrafiltered as above. Portions of the ultrafiltrate and residual solution were examined by chromatography and starch gel electrophoresis.

Acetic acid and aqueous extracts of flour from a large number of wheat species and varieties were similarly examined by starch gel electrophoresis as described in the appropriate sections.

Wedge (Zwickel) and adhering (Haft) protein (cf. Hess, 1961). Samples (0.5 g.) of wedge and adhering protein isolated from wheat endosperm by microscopic procedures were dispersed in acetic acid solution (N/100, 6 ml.) and portions of the extracts examined by starch gel electrophoresis. The adhering protein extract was extremely weak due to excessive starch dilution and had to be concentrated by freeze-drying and re-examined.

Air-classified/...

Air-classified flours. Acetic acid extracts of the following flour fractions (2.0 g./6 ml., N/100) obtained from the same parent flour by the air-classification procedure, were examined by starch gel electrophoresis.

(1) Coarse ($>35\mu$), medium (17-35 μ), fine ($<17\mu$) fractions (J. and R. Snodgrass Ltd., Glasgow).

(2) Coarse, medium, fine fractions (Spillers Ltd., London)

(3) Low (1.08% N), intermediate (1.61% N), high (2.58% N) protein fractions (Spillers Ltd., London).

Strong, medium and weak flours. Samples (2 g.) of strong, medium and weak flours (commercial classification; Spillers Ltd., London) were dispersed in acetic acid solution (N/100, 6 ml.) and portions of the extracts examined by starch gel electrophoresis.

(b) Wheat life cycle

Changes in the protein composition of the wheat seed, particularly that of the endosperm, were followed throughout the life cycle of the plant.

The investigation was conveniently divided into the following stages:

- (i) Vernalisation
- (ii) Germination
- (iii) Growth
- (iv) Ripening
- (v) Post-harvest.

(i) Vernalisation

Ripe wheat seeds (Als) were evenly spread on moist paper towelling and/...

and stored for 7 days at 4°C. After 4 to 5 days however, some of the seeds began to germinate, thus preventing an accurate assessment of any changes which might be attributable to the vernalisation procedure. A second sample was then stored in a dry state at -15°C for 7 days. During this period no germination was observed. To determine whether vernalisation had taken place, a sample of the seeds was transferred to moist paper and allowed to stand at room temperature. After 2 days the seeds began to germinate, indicating satisfactory vernalisation. Embryos were removed from the dry seeds (approx. 100) by hand dissection and dispersed in acetic acid solution (N/100 5 ml.) for 30 min. After centrifuging at 3,000 g. (0°) a portion of the extract was examined by starch gel electrophoresis. An extract of an unvernalsed sample was used as a control.

The seeds were then ground by hand and the endosperm material removed by sieving (100 mesh). Acetic acid extracts of the flour with similar extracts of unvernalsed samples were then examined by starch gel electrophoresis as above.

(ii) Germination

The early stages of growth of the plant were readily studied by germinating samples in the laboratory, thus avoiding possible attack by soil bacteria which might disturb the natural protein degradation pattern.

Seeds were initially germinated by soaking for 24 hours at room temperature followed by incubation at 25° in a thermostatted bath. After 3 days/...

days, however, the seeds developed a rancid smell and were rejected. The following method was then adopted.

Vernalised seeds (Als variety) were evenly spread on moist filter paper (Whatman No. 3) in a photographic tray (10" x 15"). A second layer of moist filter paper was placed on top of the seeds, and the sample sandwiched between two thick pads of moist cellulose wadding.* Several layers of seeds were similarly prepared. Finally, the tray was covered with a sheet of Polythene and placed in a darkened cupboard at room temperature.

Samples (approx. 100 seeds) were removed daily and immediately frozen in liquid oxygen. After removal of embryonic material, both portions of the seed were ground to a fine powder and extracted in acetic acid solution as in the previous section.

Samples of the extracts were examined by starch gel electrophoresis and by 2-dimensional chromatography after ultrafiltration as described on p. 63.

Sampling was continued for 20 days, i.e. until complete exhaustion of endosperm material. From this stage it was possible to utilise field samples for further examination. The total nitrogen content of each sample was determined by the micro-Kjeldahl technique (p. 65).

(iii) Growth

Young wheat seedlings were harvested from field plots shortly after the first/...

*Moist paper towelling used for this purpose caused significant growth of moulds.

first appearance of green leaves until flowering, as shown on p. 83. All samples were frozen in liquid oxygen immediately after harvesting.

3 Weeks after planting. At this stage the aerial parts of the plant contained only young leaves. These were removed by hand dissection and ground to a fine powder in liquid oxygen. A sample (10 g., fresh weight) was then dispersed in acetic acid solution (N/100, 30 ml.) and the extract freeze-dried.

The small amount of dried material was taken up in acetic acid solution (N/100, 0.5 ml.) and examined by starch gel electrophoresis and 2-dimensional chromatography.

A similar extraction was carried out in sodium tetraborate solution (M/20, 30 ml.) and the extract again freeze-dried. A portion of the residual material (0.5 g.) was then taken up in distilled water (6 ml.) and examined by starch gel electrophoresis in a tris/citric acid buffer (p. 54).

24-34 weeks after planting. After 24 weeks, immature spikelets approx. 1 mm. in length were visible at the growing tip of the young seedling. These were removed by hand dissection (approx. 100 seedlings), ground in liquid oxygen and dispersed in acetic acid solution (N/100, 6 ml.). After freeze-drying, the residual material was taken up in acetic acid solution (0.5 ml.) and examined by starch gel electrophoresis in an aluminium lactate/lactic acid buffer and by chromatography as before.

Leaf/acetic acid extracts were again examined as in the previous sample. Examination of leaves was discontinued from this stage.

Samples/...

Date	Weeks After Planting	Field Data	Dissection Data
15th October 1961	0	Seeds planted	-
7th November 1962	3		All aerial parts examined (leaves only)
10th April 1962	24		Immature spikelet removed
29th May 1962	31		"
18th June 1962	33½	Date of shooting (appearance of spikelet above uppermost leaf)	-
21st June 1962	34		Immature spikelet removed
25th June 1962	34½	Date of flowering (first appearance of anthers)	-
3rd July 1962	36	Estimated date of pollination	Immature seeds removed from spikelet
11th July 1962	37		"
18th July 1962	38		"
25th July 1962	39		Milky endosperm removed from seeds
1st August 1962	40		"
8th August 1962	41		"
15th August 1962	42		Embryos and endosperm removed
22nd August 1962	43		"
29th August 1962	44		"
31st August 1962	44 approx.	Date of ripening	-
5th September 1962	45		Embryos and endosperm removed
10th September 1962	46	Date of harvest (field)	"
24th September 1962	48		"
22nd October 1962	52		"

Samples were harvested 31 and 34 weeks after planting, and acetic acid extracts of the young spikelet similarly examined.

(iv) Ripening

36-38 Weeks after planting. At this stage it was possible to remove the young seeds from the wheat head. It was not possible however to further dissect the seed except perhaps by manipulation under a microscope which was not considered practical in this investigation.

Spikelets were frozen in liquid oxygen and then lightly ground with a pestle or similar heavy roller. In this way, the husks were quickly removed and the young seeds isolated intact with a pair of forceps.

The immature seeds were then ground to a fine powder and extracted in acetic acid solution. The extract was freeze-dried, taken up in acetic acid solution (N/100, 0.5 ml.) and examined by starch gel electrophoresis and chromatography as in the previous section. Samples harvested 37 and 38 weeks after planting were similarly examined.

39-41 weeks after planting. After 39 weeks, the immature seeds contained sufficient endosperm material to allow removal of the testa and associated tissues. At this stage, however, the embryonic parts were not sufficiently developed to merit removal, and could be detected only under magnification.

The endosperm material was removed from the green testa by hand dissection and dispersed in acetic acid solution (N/100, 20 ml.; approx. 100 seeds).

A portion of the extract was examined by starch gel electrophoresis in/...

in an aluminium lactate/lactic acid buffer.

Acetic acid extracts of samples obtained 40 and 41 weeks after planting were similarly examined,

42-46 weeks after planting. Immature seeds were isolated from the spikelets as described in the previous section. At this stage, the embryonic parts had developed sufficiently to allow their removal by hand dissection. The endosperm material was isolated as already described. Sample (0.1 g.) of the isolated embryos, was dispersed in acetic acid solution (N/100, 5 ml.) and a portion of the extract examined by starch gel electrophoresis as above.

An acetic acid extract (N/100, 6 ml.) of the endosperm material (10 g.) was similarly examined.

Samples were harvested 43, 44, 45 and 46 weeks after planting, i.e. until full ripening and examined as above.

(v) Post harvest

Samples obtained 2 and 6 weeks after crop harvesting and threshing were examined as above.

(c) Protein composition of various Graminae

Samples of the genera listed below were milled by hand and the flour removed by sieving (100 mesh). A portion (2.0 g.) was then dispersed in distilled water (6 ml.) as previously described and the extract examined by starch gel electrophoresis.

Samples examined were as follows:

Barley/...

Barley (Hordeum vulgare, cv. Ymer)
Oats (Avena sativa, cv. Blenda)
Rye (Secale cereale, cv. unknown)
Millet (Panicum miliaceum, cv. unknown)
Rice (Oryza sativa, cv. unknown)
Maize (Zea mays, cv. unknown)
Milo (Sorghum vulgare, cv. unknown)
Couch grass (Agropyrum repens, wild variety)
Wheat (Triticum vulgare, cv. Als)

(d) Protein composition of species of Triticum and closely related genera

The following samples were milled and aqueous flour extracts obtained as described in the previous section:

Aegilops squarrosa (v. Typica No. 2) considered to be one of the primitive ancestors of more modern wheat species.

Triticum monococcum L. (cultivated Einkorn (CI.2433), a 14 chromosome wheat, one of the most primitive of the genus, thought to be derived directly from T. aegilopoides.

T. dicoccum Schubl (Emmer) (CI.3686), a 28 chromosome wheat which has been successfully crossed with hard red spring wheat varieties and has been associated genetically with couch grass (Agropyrum repens)

T. durum Desf (cv, Mindum), a 28 chromosome wheat possessing some of the qualities of T. dicoccum, T. turgidum and to some extent T. vulgare.

T. turgidum L. (cv. 1G1), a 28 chromosome wheat closely related morphologically to T. durum.

T. polonicum/...

T. polonicum L. (cv. 7F1), a 28 chromosome wheat also closely associated with T. durum.

T. timopheevi Zhuk. (cv. 1T1), a 28 chromosome wheat related to T. durum but possessing certain distinct morphological characteristics.

T. vulgare Vill. (cv. Als), a 21 chromosome wheat widely cultivated and commonly used for bread baking.

T. spelta L. Spelt. (cv. unknown), a 21 chromosome wheat thought to be a hybrid of T. dicoccum and T. aegilopoides.

T. compactum Host (cv. 8K2), a 21 chromosome wheat resembling T. vulgare in quality.

Samples (0.2 ml.) of each extract were examined by starch gel electrophoresis. Densitometer scans were made of each pattern to facilitate comparisons.

(e) The protein composition of different varieties of T. vulgare

The following varieties of T. vulgare were milled by hand and aqueous flour extracts examined by starch gel electrophoresis as in the previous section. Densitometer scans of each pattern are shown in Fig. 27.

Champlein	Glasnevin Rosa	Gabo
Viking	Vilmorin 27	Kaw
Professeur Marchal	Maitre Pierre	Chieftain x Tenmarq
Capelle Desprez	Minister	Manitoba
Nord Desprez	Fylgia II	Russian
N59	Als	

(f)/...

(f) The protein composition of similar varieties of *T. vulgare* obtained from different sources

Samples of *T. vulgare* bred from the same cross but obtained from different sources were examined in an attempt to check the reproducibility of varietal patterns already obtained.

Aqueous extracts of the following varieties were examined by starch gel electrophoresis:

<u>Variety</u>	<u>Source (cf. materials)</u>
Champlein	Department of Agriculture, East Craigs
Viking	" " " " "
Jufy	" " " " "
Champlein	Messrs W. Kay
Viking	" " "
Jufy	" " "

(g) The protein composition of related varieties of *T. vulgare*

Closely related varieties of *T. vulgare* of known pedigree listed below were milled and aqueous flour extracts prepared as in the previous section.

<u>Variety</u>	<u>Pedigree</u>
Jufy I	Jubiligem x Fylgia
Phoebus	Jubiligem x Fylgia
Fylgia	Aurore x Extra Kolben II
Fylgia II	Aurore x Extra Kolben II
Skandia	Fylgia x Crown
Jubiligem	Vilmorin 23 x Iron III
Welcome/...	

<u>Variety</u>	<u>Pedigree</u>
Welcome	Jubiligem x Wilma
Dominator	Jubiligem x Atle
Leda	Jubiligem x Zanda
Als	Danish land variety

Samples of each extract were examined by starch gel electrophoresis.

Densitometer scans of patterns are shown in Fig. 28.

(h) The effect of plant selection on protein composition

New varieties of wheat may be introduced either by cross-breeding between two existing varieties, or by plant selection and development from a single variety. The former method has already been shown to lead to distinct changes in the protein constituents, even among varieties from the same parent forms (cf. p. 130). It was of interest therefore to examine the protein composition of varieties developed by plant selection to determine if changes in only the morphological characteristics were also reflected by alteration of the protein components.

Aqueous extracts of the following varieties of T. vulgare were examined by starch gel electrophoresis as in the previous section.

- (A) Squarehead's Master
- (B) Squarehead's Master 13/4 (plant selection from (A))
- (C) Wilhelmina
- (D) Wilma (plant selection from (C))

RESULTS

METHODS OF PROTEIN FRACTIONATION

Paper electrophoresis

Initial fractionations of wheat proteins by paper electrophoresis were comparable with those previously described in the literature (cf. Pence and Elder, 1953, Pence et al., 1954_c). Electrophoresis of sodium chloride extracts of gluten allowed the resolution of at least 8 components, while the method generally permitted satisfactory comparisons to be made of the effectiveness of various extraction procedures (cf. p. 66). The clearly defined banding of subsequent starch gel fractionations, was not however obtained, most components being detected as partly diffuse areas of more intense staining.

Cellulose acetate and agar gel electrophoresis

Fractionations on cellulose acetate were less successful than on paper. A maximum resolution of 3-4 components was obtained from a gluten-sodium chloride extract, with poorly-defined banding and considerable streaking in many cases.

Agar gels were found to be unsuitable for the fractionation of wheat protein systems under the conditions employed.

Starch gel electrophoresis

Starch gel electrophoresis was found to be by far the most effective method of fractionation of wheat proteins. By incorporation of the aluminium-lactate-lactic acid buffer system of Jones et al. (1959b) and reduction of the starch/...

starch concentration to 10%, a maximum resolution of at least 32 components was obtained from a gluten-acetic acid solution. Increased gel concentrations appeared to reduce the resolution obtained, while a 10% gel was the minimum that could be handled with ease.

Increased resolution was also obtained by staining in a Nigrosine solution. Initial experiments showed that although Naphthalene Black 12B was an effective staining agent, many of the weaker components were lost on subsequent gel washing, particularly in acetic acid solutions. A similar effect was reported by Smithies (1959a) with insulin staining.

Under the electrophoretic conditions already described (p. 50) no cooling was required for the smaller gels (8" x 4") although some curving of the patterns was observed. It was found, however, that considerable distortion of the patterns occurred if the samples were positioned less than $\frac{1}{4}$ " from the edge of the gel. Consequently, gels prepared in narrow moulds (7" x $\frac{3}{4}$ "; Messrs Shandon and Co. Ltd., London) were extremely prone to distortion by these "side effects".

In most cases, protein solutions obtained by the standard extraction procedures described were of a suitable concentration for direct electrophoretic separation. In some solvents however (e.g. N-methylformamide and N-N-dimethylformamide) normal visualisation of the faster moving components resulted in a serious overloading with respect to the slowest fractions, which necessitated a second separation at a reduced protein concentration.

The/...

The aluminium lactate-lactic acid buffer system allowed a clear resolution of the protein components of untreated extracts of flour, gluten and dormant wheat embryos, as well as most of the components of barley, rye, maize, millet, sorghum and couch grass. Some of the components of rice, millet, maize and sorghum, however, were less clearly resolved. Extracts of germinated embryos and leaves of seedlings were not fractionated under these conditions, and a tris-citric acid buffer system was employed allowing the fractionation of leaf proteins into at least 8 components.

All solutions of aluminium lactate were prepared in the laboratory as described. The use of commercial samples of aluminium lactate (Fielding Chem. Co., New Jersey) for buffer or electrolyte solutions, seriously reduced the degree of resolution.

Polyacrylamide gel electrophoresis

The method of electrophoresis in polyacrylamide gels described by Davies and Ornstein (1959) (cf. Cruft, 1962b) was applied to the fractionation of wheat proteins principally to provide an alternative medium for fractionation, but at the same time to utilise the inert qualities of the gels for detection of enzymic (particularly amylase) activity in isolated protein fractions.

Aqueous gels were initially prepared using a Cyanogum 41 Gelling agent (American Cyanamid Co., N.Y.), but these were found to polymerise irregularly and often required extremely long periods for total polymerisation. Trial fractionations of gluten-acetic acid solutions were however partially successful/...

successful allowing the separation of 4-5 sharply-defined components. The relative success of the method prompted the use of the better-defined procedure of Cruft (1962), by which gels were easily prepared incorporating the aluminium lactate buffer system used successfully for starch gel fractionations. Initially some difficulty was experienced in obtaining even polymerisation but this was overcome by ensuring the exclusion of all air bubbles from the gel mould.

Initial fractionations in 10% gels (w.r.t. acrylamide concentration) were relatively unsuccessful. Much streaking was observed and a large amount of the material appeared to be held back at the origin.

Improved fractionation was subsequently obtained with less concentrated gels. Fractionations in a 7% gel appeared to be very similar to those already obtained in starch, although the components were somewhat less clearly defined. The method was undoubtedly potentially useful, but required more development. It was therefore considered more profitable to utilise the starch gel technique than to investigate further the method at this stage.

Preparative column electrophoresis

Essentially, the aim of the column electrophoresis methods was to obtain samples of wheat protein fractions or groups of fractions in sufficiently large amounts to allow a more detailed investigation by more refined fractionation techniques.

Electrophoresis of a gluten-acetic acid solution on cellulose powder allowed very little resolution of the protein components. Recovery of material/...

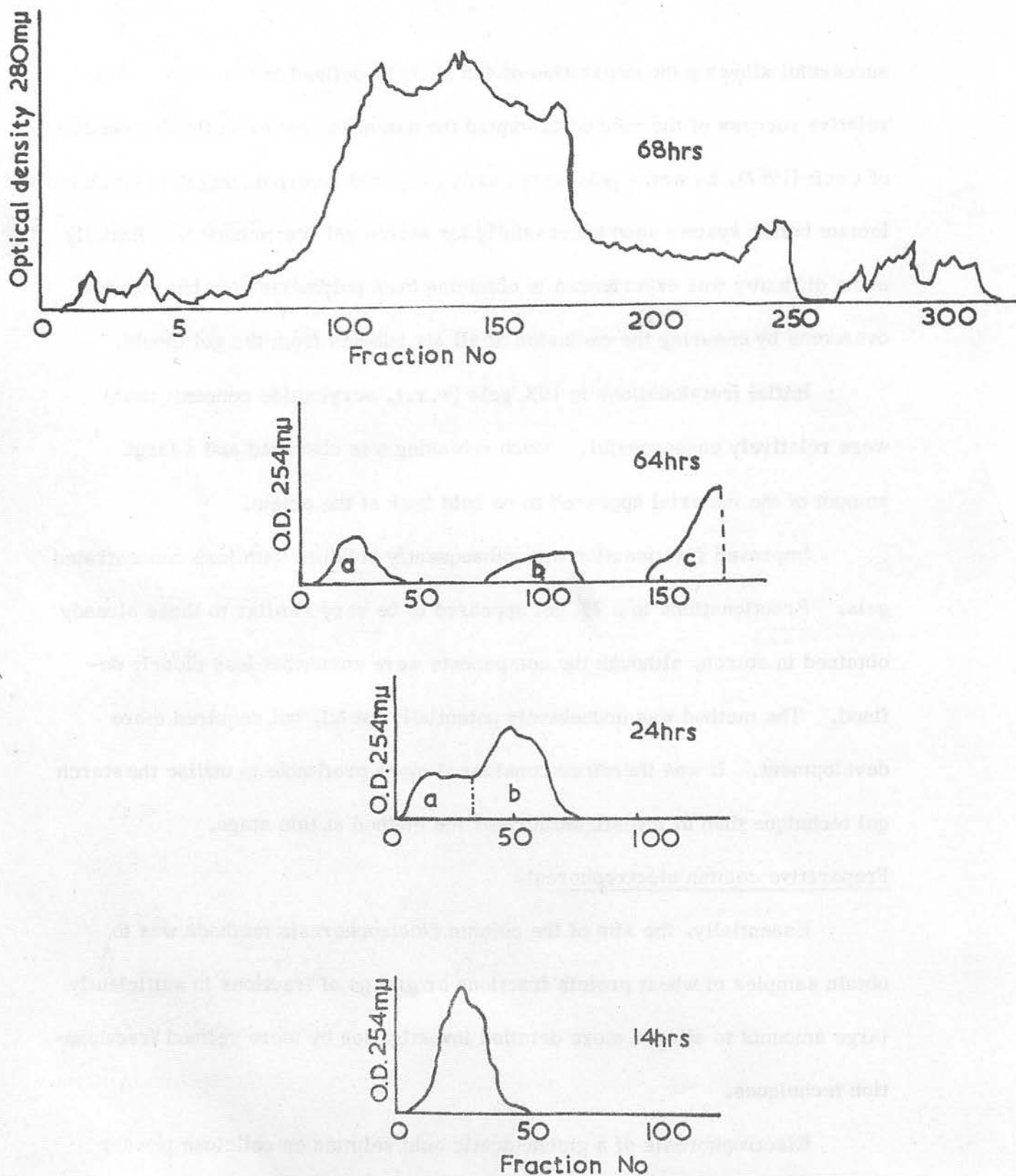


FIG. 8. Sucrose density gradient electrophoresis of dilute acetic acid soluble gluten proteins.

material was very low, indicating considerable adsorption of the migrating species, particularly since extended times of elution (up to 180 hours) were required.

Fractionations in a density gradient were, however, more satisfactory. In this case problems of adsorption were eliminated and a maximum resolution of a gluten-acetic acid solution into at least 6 components was obtained after 68 hours electrophoresis.

Fractionations carried out after 14 h, 24 h, 64 h and 68 h are shown in Fig. 8.

After 24 hours electrophoresis (Fig. 8(iii)) fractions (a) and (b) were examined by starch gel electrophoresis. Fraction (a) contained most of the components of low mobility corresponding to regions A - E in the starch gel pattern (Fig. 12) while fraction (b) contained only the major components of intermediate mobility of region F.

Fractions (a), (b) and (c) after 64 hours electrophoresis (Fig. 8(ii)) were similarly examined. Fraction (a) contained the bulk of the slow-moving components (regions A-C) as well as the major components of intermediate mobility (region F). Fraction (b) contained the remainder of the slow-moving components (regions D-E) while fraction (c) also contained components of low mobility (regions A-C) similar to fraction (a) but in a much lower concentration.

In most cases individual protein components in starch gels were poorly defined, thus preventing a more accurate assessment of the protein composition of each eluted fraction.

Molecular Sieve Methods

Electrophoresis markers for wheat proteins

Of the protein samples used as markers for wheat protein components all, with the exception of pepsin, were readily fractionated in the aluminium lactate buffer. Several of the supposedly single protein samples were, however, heterogeneous and covered a wide range of protein mobility.

The papain extract appeared to contain at least 4 fractions of similar mobility to the faster-moving gluten components (Fig. 12, regions F-G) while the bovine glycoprotein solution contained 3-4 fractions of lower mobility (region F).

Barley β -amylase contained one major fraction corresponding in mobility to the second fastest gluten component of region F and a minor fraction which corresponded to the fastest-moving gluten component of region E. It was also of interest to note that this extract caused a significant amount of degradation of the starch gel near the origin, analogous to that already attributed to β -amylase activity in wheat protein extracts (cf. p. 55).

The egg white sample contained at least 11 fractions covering the complete mobility range of gluten components.

Estimation of molecular size by membrane filtration

The characterisation of proteins on the basis of molecular size has recently received much attention. The free diffusion dialysis studies of Craig et al. (1957) have shown that molecular weight estimations of proteins may/...

may be made on the basis of their rates of diffusion through membranes of standard pore size. The method has been successfully adapted by Pierce and Free (1961) to the molecular sieving of proteins through standard membranes during starch gel electrophoresis.

A similar method of electrodialysis was used in an attempt to differentiate wheat protein components during starch gel electrophoresis and also to obtain an estimate of the molecular sizes involved by comparison with proteins of known molecular weight.

Collected results of filtrations of wheat proteins through membranes of various porosities are presented diagrammatically in Fig. 9.

Corresponding results for the following standard proteins are given below; molecular weights (Pierce and Free, 1961) are given in each case.

Bovine serum albumin	(68,000)
Bovine haemoglobin	(67,000)
Ovalbumin	(45,000)
Chymotrypsin	(24,500).

5 μ to 10 m μ filters (Millipore)

All controls passed unretarded.

60% zinc chloride treated membranes (20/32"; Visking)

Bovine serum albumin passed with some restriction.

Bovine haemoglobin passed unretarded.

Ovalbumin completely retarded.

Chymotrypsin passed unretarded.

58%/...

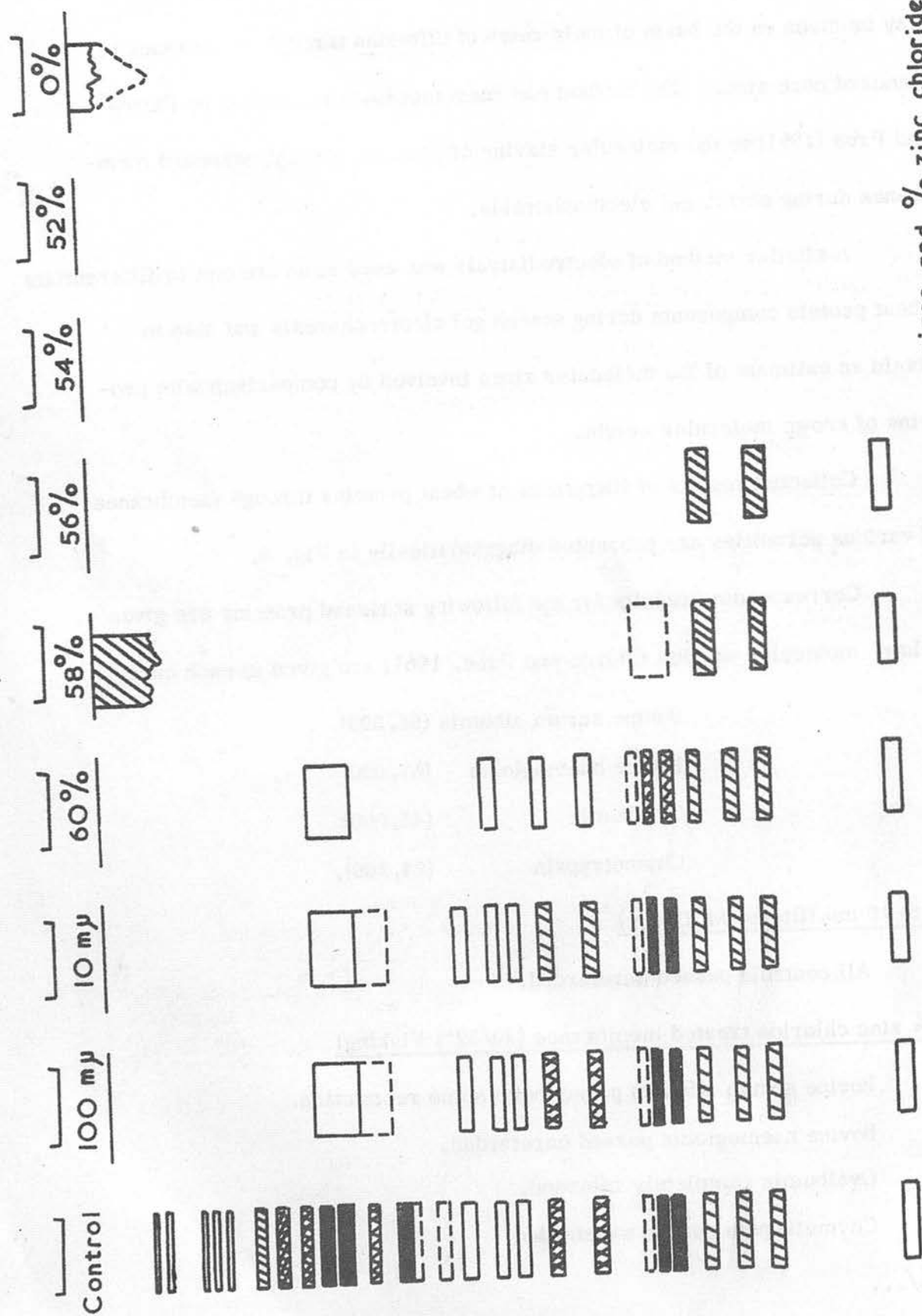


FIG. 9. Membrane filtration of wheat proteins showing average pore sizes and % zinc chloride. Filters $> 100 \text{ m}\mu$ allowed unretarded passage of all fractions.

58% zinc chloride treated membranes (20/32"; Visking)

Bovine serum albumin completely retarded.

Bovine haemoglobin passed unretarded.

Ovalbumin completely retarded.

Chymotrypsin passed unretarded.

56% zinc chloride treated membranes (20/32"; Visking)

Bovine serum albumin completely retarded.

Bovine haemoglobin passed unretarded.

Ovalbumin completely retarded.

Chymotrypsin passed unretarded.

54%, 52% zinc chloride treated membranes (20/32"; Visking)

All controls completely retarded.

Untreated membranes (20/32"; Visking)

Bovine serum albumin completely retarded.

Bovine haemoglobin passed with some restriction.

Ovalbumin completely retarded.

Chymotrypsin passed unretarded.

As can be seen from Fig. 9, all of the wheat protein components and standards passed unretarded through membranes of pore size $>100 \text{ m}\mu$. The protein components of the standard samples were clearly resolved, bovine serum albumin containing 3-4 fractions, bovine haemoglobin at least 3 fractions and ovalbumin and chymotrypsin each containing 2-3 fractions.

Membranes (Millipore) of pore size $100 \text{ m}\mu$, $50 \text{ m}\mu$ and $10 \text{ m}\mu$, while allowing/...

allowing the unretarded passage of all standards, appeared to retard completely, most of the wheat protein components of lowest mobility (Fig. 12, regions A, B and D). Some of the slower-moving components (region C) as well as all of the faster-moving components passed unretarded through the membranes. This appeared to indicate that most of the slower-moving wheat protein fractions were extremely large, of molecular dimensions $>100 \text{ m}\mu$. Some type of protein complexing may be involved in this case.

The utilisation of the swelling properties of cellophane films in zinc chloride solution allowed further differentiation of the wheat protein components.

Membranes (Visking) treated with 60% zinc chloride solution gave patterns almost identical to those obtained above ($<100 \text{ m}\mu$). All standards, with the exception of ovalbumin, passed unretarded, although slight restriction of the bovine serum albumin components was observed. It was noted, however, that the brown coloured moiety presumably associated with the haematin group did not pass through this and subsequent membranes, although the resultant protein-stained pattern was identical to the control sample. This may indicate some dissociation of the haemoglobin components at conditions of low pH. Restriction of the haematin component by the membrane may be due to ionic effects. In the control sample, the brown colour appeared to be associated with the major protein component, and this fraction did not appear to be significantly altered by its removal.

Filtration through 58% zinc chloride treated membranes appeared to further/...

further restrict several of the wheat protein components. These fractions included the slow-moving components passed by the previous membrane as well as several of the faster-moving components. A small amount of retarded material was also observed at the origin. Only bovine haemoglobin and chymotrypsin passed unretarded through the membranes. Results for bovine serum albumin filtrations are in close agreement with those of Pierce and Free (1961), who reported an unretarded passage through membranes (Visking 20/32") treated with >59% zinc chloride solution. On the basis of these results and on the standard filtrations of Pierce and Free, it seems likely that those components retarded by the latter membranes and allowed to pass unretarded through the "60%" membranes are probably of a molecular weight comparable with bovine serum albumin (68,000).

Similar patterns were obtained for wheat protein filtrations through 56% zinc chloride treated membranes, except for the removal of trace components as shown. Bovine haemoglobin and chymotrypsin components again passed through the membranes completely unretarded.

No wheat protein components or standard proteins passed through 54% and 52% zinc chloride treated membranes.

An untreated membrane (20/32"; Visking) did, however, allow the restricted passage of a small amount of wheat protein material. Again, haemoglobin and chymotrypsin components passed unretarded. This would seem to indicate that the faster-moving gluten fractions which passed through all/...

all membranes treated with >56% zinc chloride solution, are of a lower molecular weight than bovine serum albumin (68,000) but higher than chymotrypsin (24,500).

Although these results must be treated with distinct reservation, they do indicate the considerable range of molecular sizes involved in the wheat protein system. Assumptions were also made that the components of bovine serum albumin and chymotrypsin were stable under the conditions of experiment. Explanations for the anomalous behaviour of bovine haemoglobin and ovalbumin may be found in steric terms. Similar results were reported by Craig et al. (1957) for the latter system.

Several other Visking membranes were also examined for the filtration of wheat proteins (cf. Craig, et al., 1957). No correlation was obtained between porosity and tubing dimensions. Results listed below indicate, however, that some of the larger membranes may be useful for future work.

80/32" - all components passed unretarded

36/32" - fastest protein components passed unretarded

20/32" - very small amount passed near the origin

18/32" - all components completely retarded

8/32" - very small amount passed without fractionation.

Sephadex filtration

In view of the obviously large molecular weight range involved in the wheat protein system it was considered that techniques such as Sephadex filtration combined with starch gel electrophoresis might provide a useful alternative/...

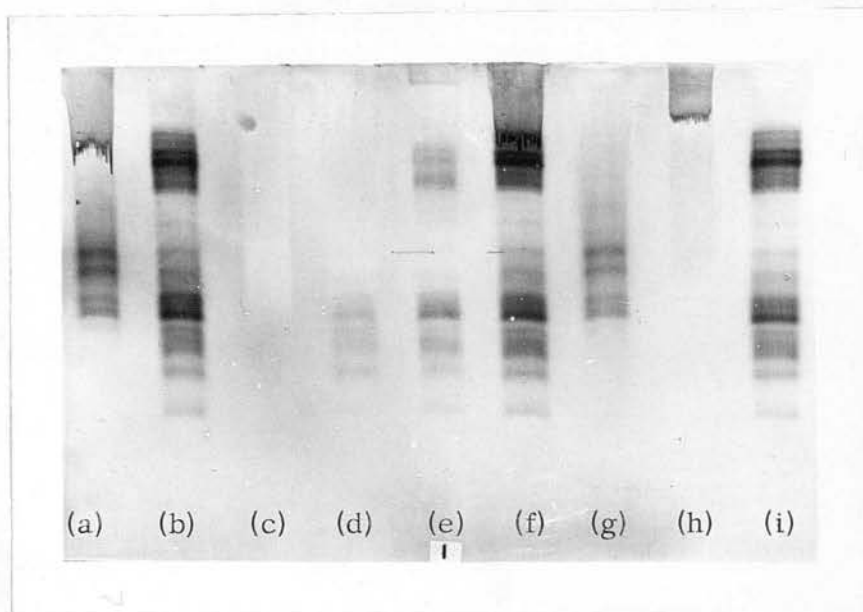


FIG. 10. Starch gel electrophoresis of water-soluble flour proteins after fractionation on Sephadex G. 100 columns (cf. Fig. 11).

Fraction 1 (h); Fraction 2 (a)(g); Fraction 3 (e);
 Fraction 4 (d); Fraction 5 (c); Untreated aqueous
 flour extracts (f); Aqueous flour extracts heated
 to 98°C (b) and (i).

alternative method of molecular weight assessment and also enable at least partial separations to be made on the basis of molecular size.

Initial filtrations were carried out using relatively small columns as described on p. 62. Subsequent filtrations on a larger scale proved to be essentially similar and, in some cases, less effective.

A series of filtrations were carried out using the various grades of Sephadex already mentioned, electrophoretic investigations being initially confined only to those components which passed unretarded through the gel matrix. No differentiation of the protein components was observed by G.25 and G.50 filtration, while grades G.75 and G.100 appeared to remove some of the faster-moving components. Only very slow flow rates were obtained using columns of G.200 and, in some cases, complete blockage occurred after sample application. The flow rate was not improved by increasing the dimensions of the column ($2\frac{1}{2}$ cm. x 20 cm.; $4\frac{1}{2}$ cm. x 20 cm.)

Further fractionations were then carried out on columns of G.75 and G.100 (Fig. 11). Little resolution was obtained on the G.75 column since most of the protein material appeared to pass through the gel relatively unretarded. A wide distribution of the protein components was obtained in most of the leading column fractions, corresponding to regions A - F of the starch gel pattern (Fig. 12). The three fastest-moving components of region F, however, appeared to be more strongly absorbed by the gel and were not visible in any of the column fractions, possibly due to excessive dilution.

Somewhat/...

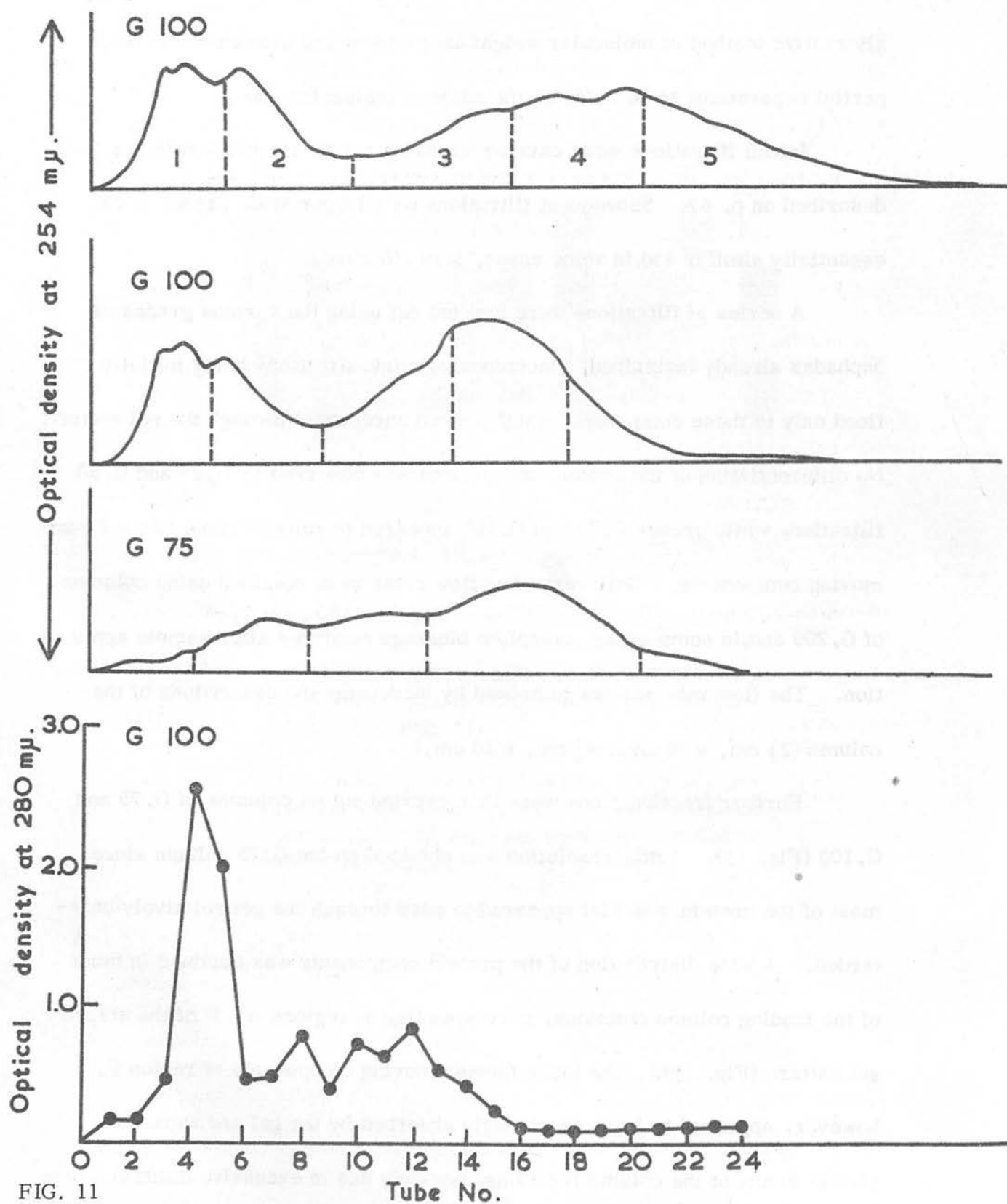


FIG. 11

Fractionation of Aqueous flour extracts by Sephadex Gel chromatography .

Somewhat better fractionations were obtained from G.100 columns (Fig. 11). Starch gel electrophoresis patterns of these fractions are shown in Fig. 10. Fraction 1 did not appear to contain any protein components resolvable in starch gel. Fraction 2 contained components similar to those excluded from the G.75 column. Fraction 3 contained a mixture of slow and fast moving components while fraction 4 contained only the latter fast-moving components. It was also noted that β -amylase activity appeared to be associated only with fractions 1 - 3 (mainly fraction 2).

Although fraction 1 absorbed strongly at 254 m μ , it was not resolvable in starch gel. One possible explanation may be that this fraction represents the extremely high molecular weight glutenin material which has been reported by others (cf. Woychik et al., 1961b) to be too large for penetration in the starch gel. This might indicate a relatively simple method of isolation of this fraction.

PROTEIN STUDIES

1. Solubility of wheat proteins

(a) Initial experiments by paper electrophoresis

The suitability of various solvent systems for the extraction of gluten proteins was initially judged on the basis of paper electrophoresis separations. Freshly extracted gluten did not appear to be readily soluble in any of the solvents examined. Lactic acid and acetic acid extractions gave essentially/...

FIG. 12

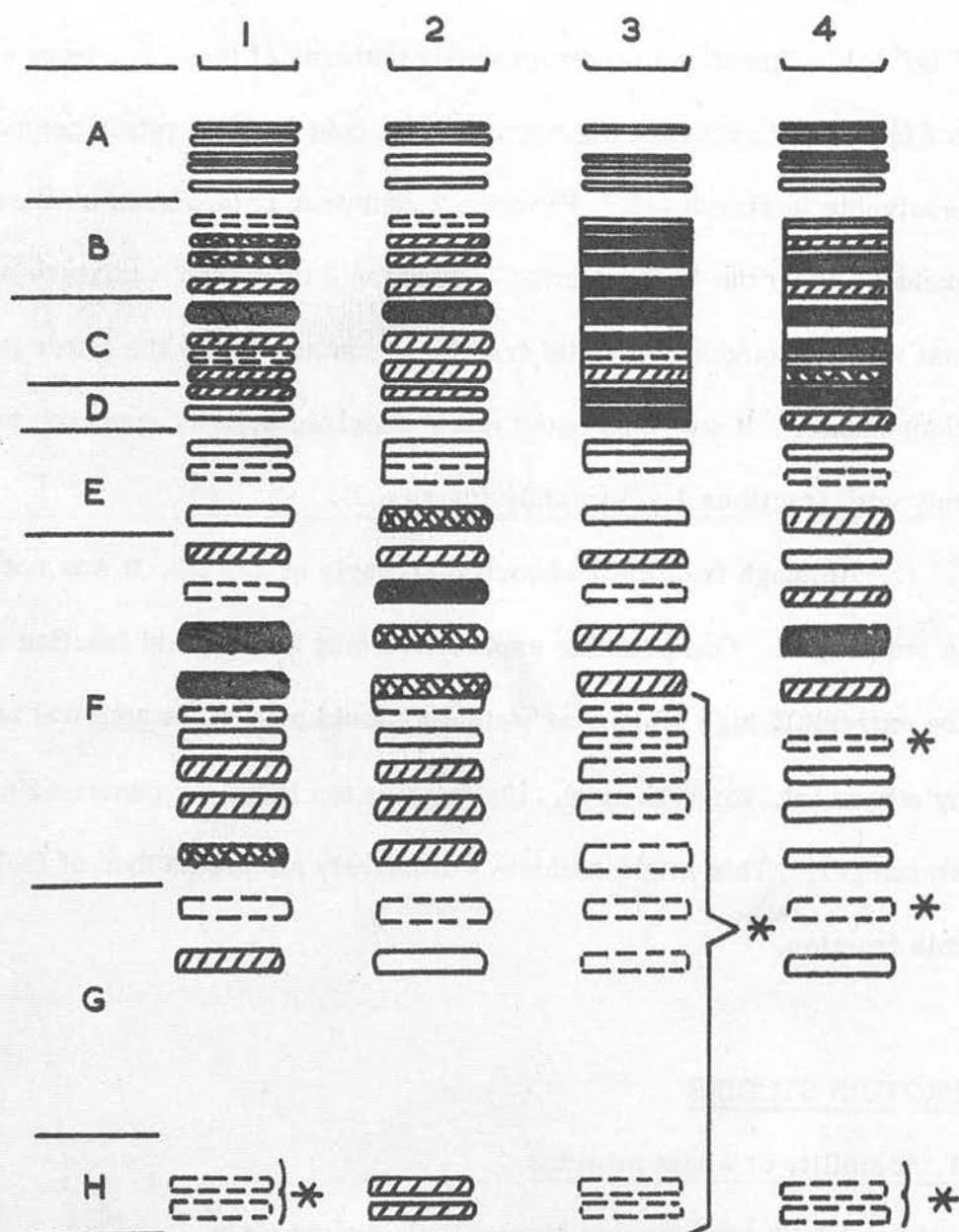


Diagram of starch gel electrophoresis patterns of components extracted in. 1. Distilled water (pH 5.6). 2. Sodium chloride (0.5 M). 3. Aqueous ethanol (70%). 4. Acetic acid (0.01 N).

* Components visible only after concentration.

essentially similar results with a maximum resolution of 5-6 readily discernable components of relatively low electrophoretic mobility. The use of formic acid, however, led to gel formation during the extraction, with considerable streaking of the electrophoresis pattern (cf. Meredith, et al., 1960a). The addition of EDTA to the solvents appeared to reduce the amount of extracted material, while acetyl acetone had the reverse effect, without alteration of the electrophoretic pattern.

Sodium salicylate extracts appeared to contain a relatively large amount of protein material, most of the components being of higher electrophoretic mobility than those extracted in acid solution. A maximum resolution of seven components was obtained, three of which corresponded to components of low mobility present in acid extracts.

Very little protein material was extracted in dilute sodium tetraborate or dilute sodium hydroxide solutions. The latter extract was resolved into two rather diffuse components of low electrophoretic mobility while the former showed little or no resolution.

(b) By starch gel electrophoresis

Much improved fractionations of flour and gluten extracts were obtained by starch gel electrophoresis in an aluminium lactate-lactic acid buffer. A diagrammatic representation of the fractionations is given in Fig. 12.

(i) General solubility

Aqueous extracts of flour contained a complete cross-section of all of the/...

the components extracted in subsequent solvents. The two components of highest mobility (region H) were, however, extremely weak and were only visible after concentration of the extract. Resolution of at least 30 components was obtained.

Fractionation of sodium chloride extracts of flour were essentially similar except that the relative solubilities of individual components appeared somewhat different. More protein material was extracted at lower salt concentrations (<0.5 M), although the general concentration distribution was identical for all extracts. In contrast to aqueous extracts, components of intermediate to high mobility (regions F to H) appeared to be preferentially soluble in salt solutions, particularly the two fastest-moving components (region H). The slower-moving fractions (A to D) were identical to the corresponding components of aqueous extracts.

Aqueous ethanol extracts of flour appeared to contain relatively few of the faster-moving protein components in contrast to the high concentration of slow-moving fractions (regions A to D). Most of the missing components of higher mobility were however observed by employing more concentrated solutions but this led to serious overloading and streaking of the slower fractions.

The distribution of components in acetic acid extracts appeared to be somewhat similar to that in ethanol, the bulk of the components being of low mobility (regions A to D). A larger proportion of the faster-moving fractions were however extracted in acid solution, in different concentrations from those in other solvents.

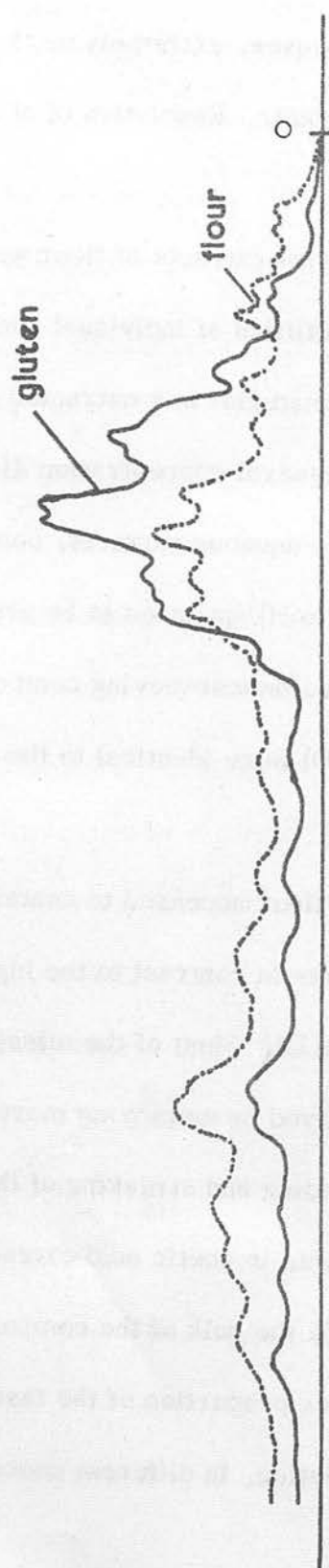


FIG. 13. Starch gel electrophoresis of the water-soluble proteins of flour and isolated gluten.

Similar results were obtained for freshly isolated gluten extracts, except that in all cases, direct dispersion of gluten led to an increased extractability of the components of low electrophoretic mobility.

A comparison of protein components of aqueous extracts of flour and an equivalent amount of isolated gluten is shown in Fig. 13.

(ii) Solubility in sodium pyrophosphate solution

Exhaustive extraction of wheat proteins in sodium pyrophosphate solution following the method of Simmonds and Winzor (1961) showed that although most of the components of intermediate electrophoretic mobility were preferentially removed, a significant amount still remained in the residual material and were extracted by subsequent acetic acid dispersion.

Initial sodium pyrophosphate extracts appeared to contain mostly components of region F (Fig. 12) similar to those preferentially extracted in sodium chloride solutions. Extracts however also contained a significant proportion of the slower moving fractions (regions A to D). Subsequent extracts contained progressively less protein material until finally no components were observed in the fourth extract.

Acetic acid extracts of the residual material contained mostly components of low mobility (regions A to D). Significant amounts of material of intermediate (region F) and higher mobility (region H) were also present however in the first and second extracts.

Most of the components extracted in pyrophosphate and acetic acid solutions/...

solutions corresponded to components present in aqueous flour extracts.

(iii) Group separation of flour proteins

A group separation of flour proteins following classical extraction procedures gave essentially similar results to those obtained in section (1).

Fractionations of aqueous flour extracts were identical with those previously obtained, and contained a complete "spectrum" of all components with the exception of the two fastest fractions which were present only in trace amounts.

Sodium chloride extracts appeared to contain most of the components previously extracted in water, with a similar distribution to that obtained by direct salt extraction.

Subsequent aqueous ethanol extracts contained significantly less protein material than obtained by direct extraction, although components of low electrophoretic mobility again appeared to be preferentially extracted. The resolution of these fractions was notably low at this concentration due to considerable streaking of the material.

2. Effect of dielectric constant on solubility

The solubility of gluten proteins in solvents of varying dielectric constant was examined in an attempt to determine whether the protein fractions already obtained by starch gel electrophoresis were true molecular species or merely loosely bound intermolecular complexes of relatively few molecular subunits.

All solvents examined appeared to increase the solubility of protein material/...

material without significant alteration of the electrophoresis patterns. Generally on the basis of protein stain intensities solubility increased with increasing dielectric constant of the solvent.

Fractionations of urea and N-N-dimethylformamide extracts were similar to those of acetic acid extracts except that they contained a larger proportion of the slower-moving components (Fig. 12, regions A to D).

A large amount of protein material was extracted in formamide and N-methylformamide which initially led to serious overloading and streaking of the electrophoresis pattern, particularly with the latter solvent. Subsequent dilution of the extract gave resolutions similar to those already obtained.

3. Protein components of commercial gluten samples

The protein components of commercially available dried gluten samples were examined as a preliminary to the study of gluten from known wheat varieties. The investigation also allowed some comment on the effectiveness of industrial methods of protein isolation and drying procedures.

The overall distribution of protein components as judged by electrophoresis patterns was similar in all acetic acid extracts, although the Vicrum extract appeared to contain a high proportion of components of intermediate electrophoretic mobility. Quite distinct differences, both quantitative and qualitative, were however observed between individual components of the low mobility group (Fig. 12, regions A to D). Qualitative differences were confined mainly to the leading fractions of the main group (region D) although some minor/...

minor differences were also observed in the slowest fractions, (region A).

The overall protein distribution in all extracts was essentially similar to that of acetic acid extracts of freshly isolated gluten.

4. Protein components of isolated gluten fractions

The protein components of isolated gluten fractions were examined, to determine the effectiveness of the isolation procedures, particularly in view of the laboratory fractionations already described.

(a) Commercial samples

Acetic acid extracts of both commercial gliadin samples contained mostly components of low electrophoretic mobility. One extract (BDH Ltd.) was very poorly resolved, with much streaking, while the other (Mann Res. Labs.) was finely resolved and contained a range of components corresponding to regions B to E and the four slowest components of region F (see Fig. 12). It was noted that the texture of these samples was considerably different, the former being brownish and granular and the latter a fine white powder.

Acetic acid extracts of glutenin (BDH Ltd.) contained no acetic acid soluble components which could be fractionated in starch gel.

(b) Precipitated fractions

Acetic acid extracts of gliadin from Lutescens-329 contained only components of low mobility (Fig. 12, regions A to D), while the corresponding extracts of variety M-2 (Lutescens-329 x Agropyrum glaucum) contained components of low and intermediate mobility (regions A to F). Distinct differences were/...

were observed between the components of low mobility particularly in regions B and D. Although the fast-moving fractions of variety M-2 were rather weak, they appeared to have a distribution of protein components which was not typical of other varieties. (Fig. 27 (v)).

5. Physical treatment of wheat proteins

The solubility of gluten in dilute acetic acid solution has for some time been recognised as a measure of the state of denaturation of the protein material (cf. Schaeffer, et al., 1960). Hence, by examination of the acetic acid soluble material from treated samples, it was hoped to obtain some assessment of the degree of denaturation and the degree to which individual components were affected by standard methods of protein denaturation.

(a) Heat stability

Samples of freshly isolated gluten when heated at 100°C, tended to form a crust which acted as an effective barrier to further drying. Thus samples heated for relatively short periods (30-60 min.) still contained a relatively large proportion of moist gluten. Acetic acid extracts of these glutes were identical to untreated controls. Complete drying, however, appeared to insolubilise the protein material. Similar results were obtained for commercially dried gluten samples.

The heating of gluten-acetic acid dispersions for short periods at 98°C did not appear to affect the extractability of the protein components.

Gluten solutions, when heated to 98-100°C for short periods (1-2 min.) tended to become hazy. This, however, did not appear to affect the protein fractionation/...

fractionation to any extent. A small amount of the precipitated material was removed by centrifugation (25,000 g.) but paper pulp filtration was required to remove the remainder.

Heating for periods of 10-30 minutes tended to cause significant precipitation and coagulation of the protein material. Subsequent fractionation of the centrifuged extracts showed a large depletion of the fractions, particularly of the slow-moving components.

As was noted before, heating to 98-100°C (1-2 min.) effectively reduced β -amylase activity.

(b) Ultrasonic treatment of gluten proteins

The effect of ultrasonic treatment of gluten was examined to determine its effectiveness as a method of protein dispersion and as a possible method of protein alteration which might lead to changes in the rheological properties of the isolated complex.

As a method of protein dispersion, it was relatively ineffective. Partly dispersed gluten particles tended to associate and become granular. Only a small amount of protein material was extracted after 60 minutes treatment (20 W/cm²) with no obvious changes in the protein composition, as judged by starch gel electrophoresis. A constant shearing force (cf. maceration, mastication) appeared to be required for adequate gluten dispersion.

(c)/...

(c) γ -irradiation of gluten, flour and wheat seeds

γ -Irradiation has been shown to cause significant alteration in the rheological properties of flour doughs and isolated gluten (cf. Deschreider, 1961; Doguchi et al., 1959; Vakar and Tolchinskaya, 1960). The nature of these changes appears largely dependent on the level of irradiation, although the effect on individual protein components is not clearly understood.

An attempt was made to assess these changes in terms of the alteration of protein components of isolated gluten, flour and dormant seeds at different levels of irradiation.

Samples treated with 8 K rad showed no alteration of the acetic acid soluble protein components. Electrophoresis patterns of all treated and untreated extracts were identical, including starch gel degradation by β -amylase action.

Higher levels of irradiation (32.5×10^6 rad) however caused drastic alteration of the protein components. The bulk of the extracted material was of low electrophoretic mobility and unresolved. Some fractionation was observed in extracts of flour from freshly-milled irradiated seeds. Only this extract appeared to possess any β -amylase activity. The faster-moving components were significantly absent in all extracts.

(d) Mechanical effects on gluten

There is considerable evidence to suggest that the gluten complex is a work-sensitive system. Much information is available concerning its effect on/...

on the rheological properties of doughs and isolated gluten, but whether these effects may be detected on a molecular level, in terms of protein alteration, remains open to speculation.

A series of freshly isolated gluten samples was subjected to varying degrees of mastication and the protein components examined by starch gel electrophoresis. In this way, it was hoped to detect any alteration of the protein fractions, although the obvious rheological changes may not necessarily be reflected by changes in individual components in solution.

All samples showed a distinct decrease in elasticity with increasing time of mastication. Extracts of samples treated for 10 and 20 minutes were not significantly different for untreated controls. Mastication for longer periods (30, 60 min.) however appeared to result in an increased solubility of the fractions of low mobility giving a component distribution somewhat similar to that of ethanol extracts. (cf. Fig. 12).

Similar effects were observed by altering the size of maceration jar used for gluten dispersion. A decrease in jar volume without alteration of amounts of gluten or solvent led to the extraction of an increased proportion of the slower-moving components.

(e) Effect of dough ageing on protein solubility

Before isolation of gluten from flour, a period of dough ageing is required to allow hydration and aggregation of the protein molecules to form the gluten complex. In view of the solubility of flour and isolated gluten proteins, particularly in water (cf. p. 103), it was considered that this ageing period might/...

might bear some relationship to the extractable protein material.

Dough formation and ageing did not appear to affect the protein solubility. Fractionations of all dough extracts were identical to those of aqueous flour extracts, irrespective of the dough ageing period. Isolated gluten extracts however again showed distinct differences in component distribution with a large increased extraction of the components of low mobility. Starch slurries contained a full quota of protein components and were similar to aqueous flour extracts, although considerably weaker.

(f) Ageing of protein solutions

Initially, protein solutions were stored at +4°C in preference to deep freezing, since repeated freezing and thawing is known to cause alteration of most proteins in solution. It was suspected however that this method of preservation caused some alteration of the protein components. Examination of samples stored for periods of 24 hours to 14 days showed that significant precipitation had occurred, particularly after 2-3 days. Only a small proportion of this material was redissolved on heating. Subsequent electrophoretic fractionation showed a distinct reduction in the concentration of components of low mobility with an apparent increase in concentration of the faster-moving components. This was particularly noticeable after 14 days ageing. No qualitative changes in protein composition were observed.

Similar, but less pronounced, effects were also noted when solutions were allowed to stand for several days at room temperature. Standing for longer/...

longer periods under these conditions, however, led to significant alteration of the protein components presumably due to bacterial action.

6. Chemical treatment of wheat proteins

(a) Lipid removal

Gluten is known to contain roughly 8% lipid material, some of which is thought to be bound to protein in the form of lipoprotein complexes (cf. Zentner, 1960). It was considered that this type of complexing might account in part for the heterogeneity of protein extracts, particularly since other workers, using "lipid-free" gluten, have reported fewer fractions under similar conditions.

Exhaustive lipid extraction of flour however led to a complete loss of dough-forming capacity. There appeared to be no obvious alteration of the acetic acid soluble protein components as judged by starch gel electrophoresis. Fractionations were essentially similar to those obtained from corresponding extracts of untreated flour.

After partial lipid removal the flour still retained its dough-forming capacity. Careful washing in minimum quantities of water was, however, required for gluten isolation since the less cohesive dough tended to disperse easily in the wash water. The treatment did not appear to alter the acetic acid soluble components, the electrophoresis patterns obtained being identical to those above.

(b)/...

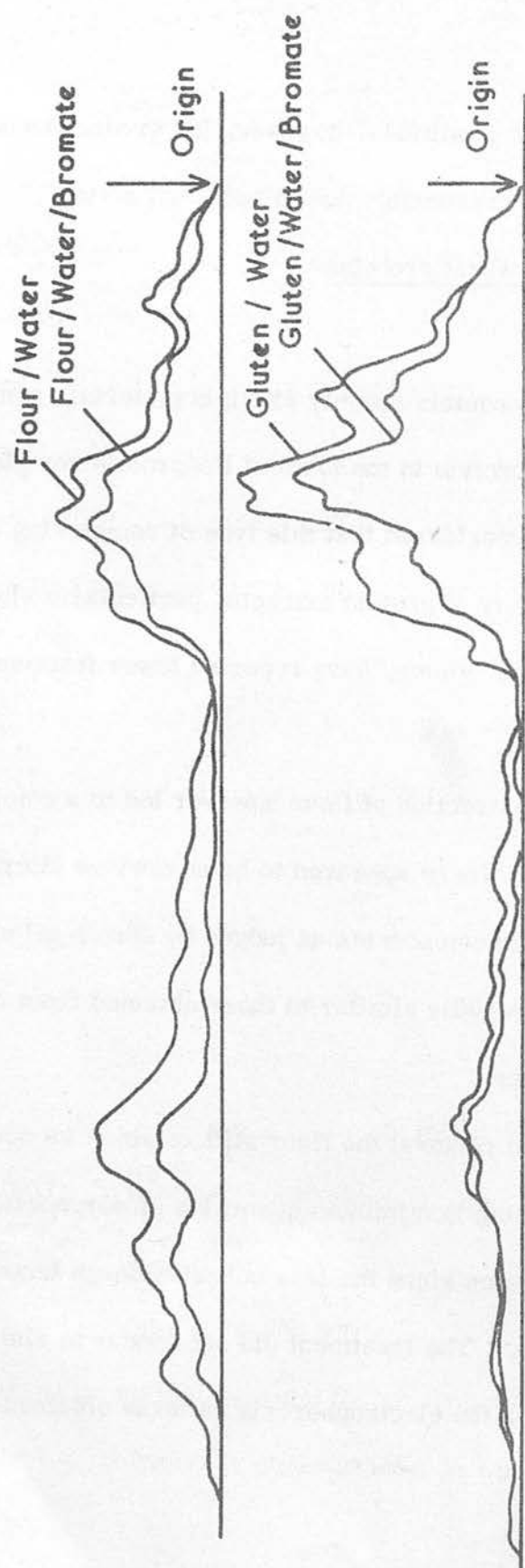


FIG. 14. Starch gel electrophoresis of aqueous flour-dough and isolated gluten extracts showing the effect of potassium bromate inclusion (60 p. p. m.) during dough preparation.

(b) Effect of redox systems on wheat proteins

Small amounts of oxidising and reducing agents are known to cause significant alteration of the physical characteristics of doughs, presumably by way of their effect on the protein components of gluten or associated substances, e.g. lipids. In an attempt to determine the extent of these changes, flour samples were treated for varying periods with oxidising agents, e.g. potassium bromate and iodate and reducing agents, e.g. cysteine and glutathione (known to be present in wheat) with subsequent examination of the protein components.

The inclusion of small amounts of oxidising and reducing agents in doughs, while causing some alteration of the rheological properties, had no appreciable effect on the extractable gluten protein components, as judged by starch gel electrophoresis (cf. Fig. 14). Doughs incorporating potassium bromate and iodate (60 p.p.m.) were noticeably shorter after ageing, while the effect of cysteine and glutathione was less noticeable.

Considerable alteration of the aqueous soluble protein components was however observed after periods of incubation (Fig. 15). Oxidation appeared mainly to reduce the amount of extractable protein material particularly those fractions of low electrophoretic mobility (Fig. 12, regions B to D), while those fractions of lowest (region A) and intermediate mobility (region F) were relatively unchanged. Similar patterns were obtained from both oxidised samples although these effects appeared to be slightly more marked in potassium/...

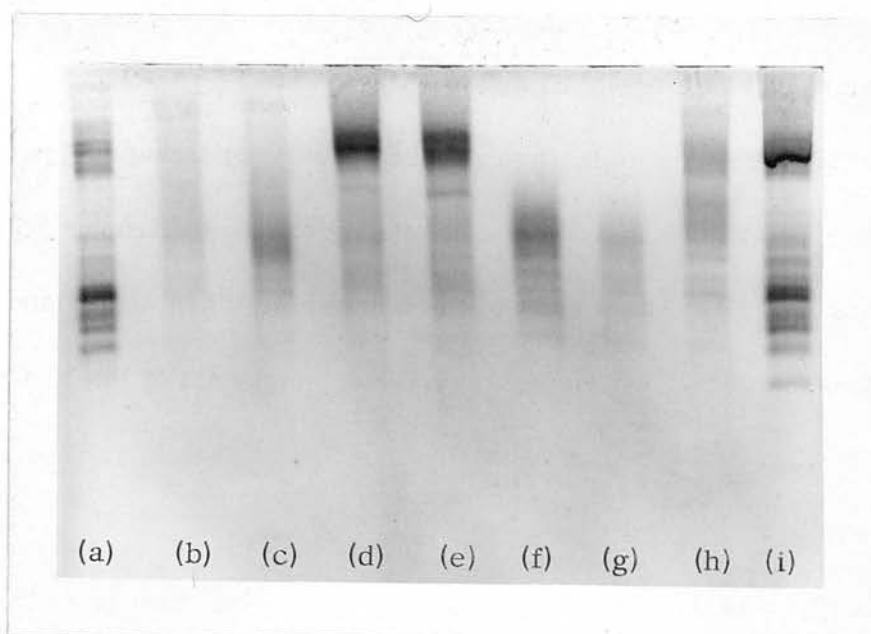


FIG. 15. Starch gel electrophoresis of water-soluble flour proteins, showing the effects of oxidation, reduction and proteolysis after 72 h incubation. (a)(i) Untreated controls, (b) + potassium iodate, (c) + potassium iodate, (d) + glutathione, (e) + cysteine, (f) + papain, (g) + papain/cysteine, (h) incubated control.

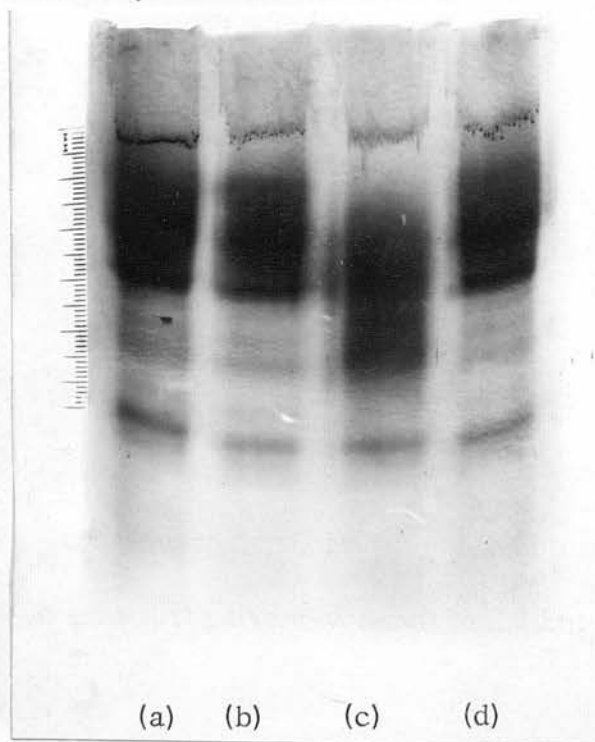


FIG. 16. Starch gel electrophoresis of dilute acetic acid soluble gluten proteins showing the effects of proteolytic enzymes after 72 h incubation. (a) Untreated control, (b) + pepsin, (c) + papain/cysteine, (d) + trypsin.

potassium bromate treated flours.

In contrast to oxidation, incubation with reducing agents appeared to increase the solubility of most components of low electrophoretic mobility (regions A to D). Again most of the components of intermediate mobility appeared unaltered although several new components of low and high electrophoretic mobility were also observed. The effects of cysteine and glutathione were essentially similar.

The effects of oxidation and reduction were obvious after 24 hours incubation and more pronounced after longer periods. Some alteration of the untreated control samples was observed after 72 hours, but this was slight in comparison with treated samples.

7. Biochemical treatment of wheat proteins

(a) Proteolytic enzymes

(i) Partial hydrolysis

The proteolysis of wheat proteins was investigated as a preliminary to "fingerprinting" experiments and germination studies and also in view of the changes which were shown to occur in protein extracts on standing.

Of the enzymes investigated, only the papain/cysteine system produced significant alteration of the protein components, even after 72 hours incubation. Significant degradation of the slow-moving fractions occurred in the papain-treated sample after 24 hours. This was more marked after 48 hours, and after 72 hours incubation few of these fractions remained. The degradation process/...

process was accompanied by the formation of new components of increasing electrophoretic mobility. As in the previous section, components of intermediate mobility appeared to be relatively unaltered. (Fig. 16).

Results for cysteine-treated samples were similar to those already obtained (section 6(b)).

(ii) Protein "fingerprinting"

The process of protein "fingerprinting" involves the partial hydrolysis of protein material with subsequent separation of the peptides produced by electrochromatography. In this way, patterns characteristic of particular proteins may be obtained in terms of peptide fragments from the protein periphery. The method was applied to various samples of gluten obtained from different wheat sources in an attempt to obtain a simple method of differentiation.

Using a papain/cysteine system optimum electrochromatographic separations were achieved in a dilute acetic acid buffer after 8 hours hydrolysis. Very little electrophoretic separation of the ultrafiltrate components was obtained in a pyridine/acetic acid/water buffer. Significant differences were observed in "fingerprints" obtained from different glutes. In most cases, fairly good reproducibility was obtained, although some minor variations occurred presumably due to the complexity of the protein systems and difficulty in obtaining absolutely standardised conditions.

(b)/...

(b) Phospholipases and amylases

Gluten is known to contain a relatively large amount of phospholipid material, which is thought by some to play an important role in the structural properties of the gluten complex (cf. Grosskreutz, 1960).

Incubation of gluten with phospholipase D has been shown to cause a marked rapid alteration of the rheological properties of the isolate (cf. Coulson and Somerville, unpublished). Similar effects were also observed by incubation with α -amylase.

It was of interest therefore to examine these treated glutes to determine if changes in the rheological properties were accompanied by corresponding changes in the protein constituents.

Similar results were obtained from both samples. No significant alteration of the protein components was observed, although the treatment appeared to substantially increase the amount of extractable protein material somewhat analogous to reduction effects.

8. Biological techniques

(a) Protein composition of the mature wheat seed

Pericarp

Acetic acid extracts of husks from ripe wheat seeds did not appear to contain any protein material as judged by starch gel electrophoresis.

Chromatographic separation of ninhydrin-positive substances showed the presence of the usual amino acids present in plant materials. Major amino acids/...

acids, judged by colour intensities, appeared to be glutamic acid, alanine, glutamine, γ -amino-n-butyric acid, leucine and probably citrulline.

Estimations were based on at least 4 separate extractions, chromatographed in triplicate.

Testa and aleurone layer

Isolated testa material appeared to contain a small amount of acetic acid soluble proteins, all of which corresponded to fractions previously observed in corresponding flour extracts. Most of these components were of intermediate electrophoretic mobility and may possibly represent a contamination by endosperm material during the isolation procedure. Ultrafiltrates again contained the usual amino acids, the major components being glutamic acid, asparagine, glutamine, γ -amino-n-butyric acid and probably citrulline.

Embryo

Embryonic material contained a large proportion of acetic acid soluble proteins, readily fractionated by starch gel electrophoresis. At least 13 fractions were observed of intermediate to high mobility, roughly corresponding to regions F and G of the endosperm protein pattern (Fig. 12). All extracts contained one major fraction in very high concentration of similar mobility to the slower-moving major fraction of region F.

Comparisons of embryonic and endosperm components over several separations indicated that the protein composition of each extract was quite different.

Similar/...

Similar fractionations were obtained for acetic acid extracts of commercial wheat germ samples.

Glutamic acid, alanine, glutamine and γ -amino-n-butyric acid appeared to be the major ninhydrin positive constituents of embryo ultrafiltrates.

Endosperm

Electrophoretic separations of acetic acid soluble endosperm proteins are described in detail under separate headings.

Chromatographic separation of ninhydrin-positive substances of endosperm ultrafiltrates revealed the presence of glutamic acid, alanine, asparagine, glutamine, proline and probably citrulline as major constituents.

Wedge (Zwickel) and adhering (Haft) protein

Acetic acid extracts of wedge and adhering protein appeared to contain a very different complement of protein fractions. Wedge proteins mainly corresponded to endosperm components of low electrophoretic mobility (Fig. 12, regions A to D) while adhering proteins corresponded to the slowest-moving endosperm components (region A) with faster-moving minor components in region F. A comparison of components of both extracts is shown in Fig. 17.

Air-classified flours

Commercial separation of flour fractions on the basis of particle size has been based essentially on the fractionation technique of Hess mentioned above. Fractions of different protein content are readily obtained by this method, /...

method, differences being attributed to the amounts of wedge and adhering protein associated with each fraction. It was of interest therefore to examine these samples in relation to the fractionations of wedge and adhering protein already obtained.

No differences were observed in the acetic acid soluble components of each flour fraction, all patterns being identical with that from the parent flour.

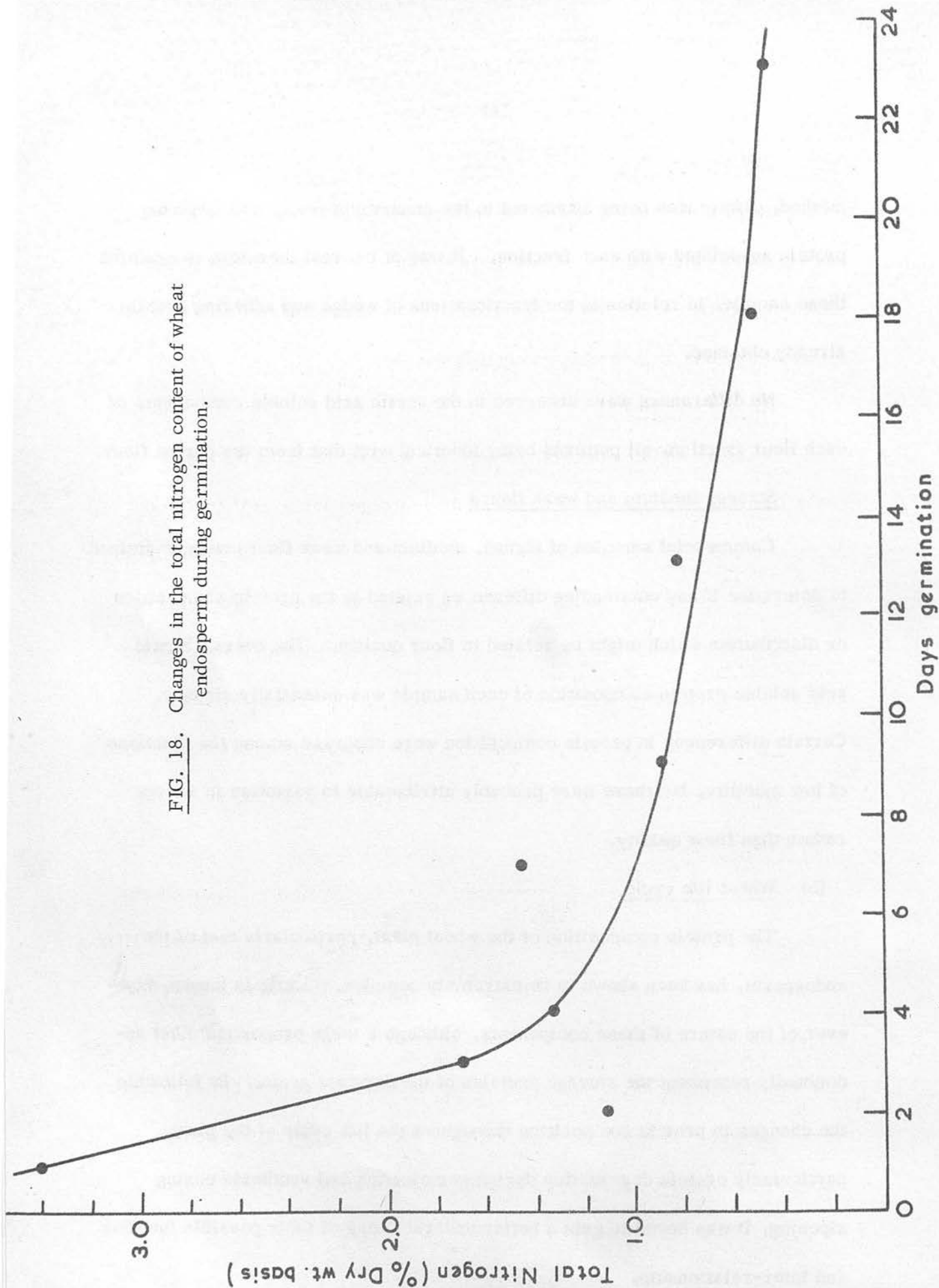
Strong, medium and weak flours

Commercial samples of strong, medium and weak flours were examined to determine if any outstanding differences existed in the protein composition or distribution which might be related to flour quality. The overall acetic acid soluble protein composition of each sample was essentially similar. Certain differences in protein composition were observed among the fractions of low mobility, but these were probably attributable to variation in source rather than flour quality.

(b) Wheat life cycle

The protein composition of the wheat plant, particularly that of the endosperm, has been shown to be extremely complex. Little is known, however, of the nature of these components, although a large proportion must undoubtedly represent the storage proteins of the dormant grain. By following the changes in protein composition throughout the life cycle of the plant, particularly protein degradation during germination and synthesis during ripening, it was hoped to gain a better understanding of their possible function and inter-relationship.

FIG. 18. Changes in the total nitrogen content of wheat endosperm during germination.



(i) Vernalisation

Storage of dormant seeds for 7 days at -15°C did not appear to cause any significant alteration of the acetic acid soluble protein components of either endosperm or embryo. Electrophoresis patterns of both extracts were identical to those of unvernalsed control samples.

(ii) Germination

Although significant degradation of endosperm proteins during the early stages of germination was indicated by nitrogen analyses (Fig. 18) no corresponding alteration of the acetic acid soluble components was observed by starch gel electrophoresis. Patterns identical to those of ungerminated controls were obtained during the first 5 days of germination. After 6 days, however, significant weakening of the slow-moving fractions (Fig. 12, regions A to D) was observed. These fractions became progressively weaker throughout the 6th to 9th days, after which only traces of protein material were observed in this region.

Fractions of higher electrophoretic mobility appeared to be unaltered throughout this period, up to approximately the 13th day of germination. After this stage, these components also became progressively weaker, until after 17 days, only traces of protein material remained. No protein material was observed in subsequent extracts. Few new components of higher mobility were observed throughout the degradation of the slow-moving fractions, although a small amount of unresolved material of higher mobility (region E) was/...

was noted between 6th and 9th days. An accumulation of protein material similar to that produced by proteinase degradation (cf. p. 116) did not however occur in this region.

Examination of incised embryos indicated that unaltered acetic acid soluble protein material could be extracted only during the first or second day of germination, after which there appeared to be a change-over to material of different solubility characteristics. Attempts to determine more accurately the point of change-over were hindered by the difficulty in determining which seeds were in the process of germinating.

All subsequent samples of embryonic material appeared to contain no acetic acid soluble components as judged by starch gel electrophoresis.

No qualitative changes were observed in the free amino acid contents of endosperm and embryo ultrafiltrates throughout germination. The major free amino acids of endosperm ultrafiltrates appeared to be alanine, glutamine, proline, γ -amino-n-butyric acid, valine-methionine and leucine. Embryo ultrafiltrates contained glutamic acid, alanine, asparagine and glutamine as major constituents.

Some variation in the levels of glutamic acid, asparagine, glutamine and γ -amino-n-butyric acid was detected in all samples throughout germination.

(iii) Growth

3 weeks after planting

Immature leaves harvested 3 weeks after planting appeared to contain
no/...

no acetic acid soluble protein material. Chromatographic separation of the extracts however showed a relatively high proportion of ninhydrin-positive material. Most of the components corresponded to the usual plant amino acids, including glutamic acid, serine, alanine, asparagine, glutamine and γ -amino-n-butyric acid as major constituents.

Sodium tetraborate extracts were shown to contain at least 8 distinct components, although a large proportion of the material was poorly resolved.

24, 31 and 34 weeks after planting

Immature spikelets removed from the plant before the date of flowering appeared to contain no acetic acid soluble protein material as judged by starch gel electrophoresis. Fractionations of sodium tetraborate soluble leaf proteins were essentially similar to those already obtained.

The free amino acid content of all acetic acid extracts was similar to that above, except for a lower level of serine and γ -amino-n-butyric acid.

36, 37 and 38 weeks after planting

Extracts of intact immature seeds harvested 36 and 37 weeks after planting showed no indication of acetic acid soluble protein components. Major ninhydrin-positive constituents were glutamic acid, alanine, asparagine, glutamine, probably citrulline and proline.

After 38 weeks however, a small amount of acetic acid soluble material was extracted from the intact seeds. These components, although extremely weak,...

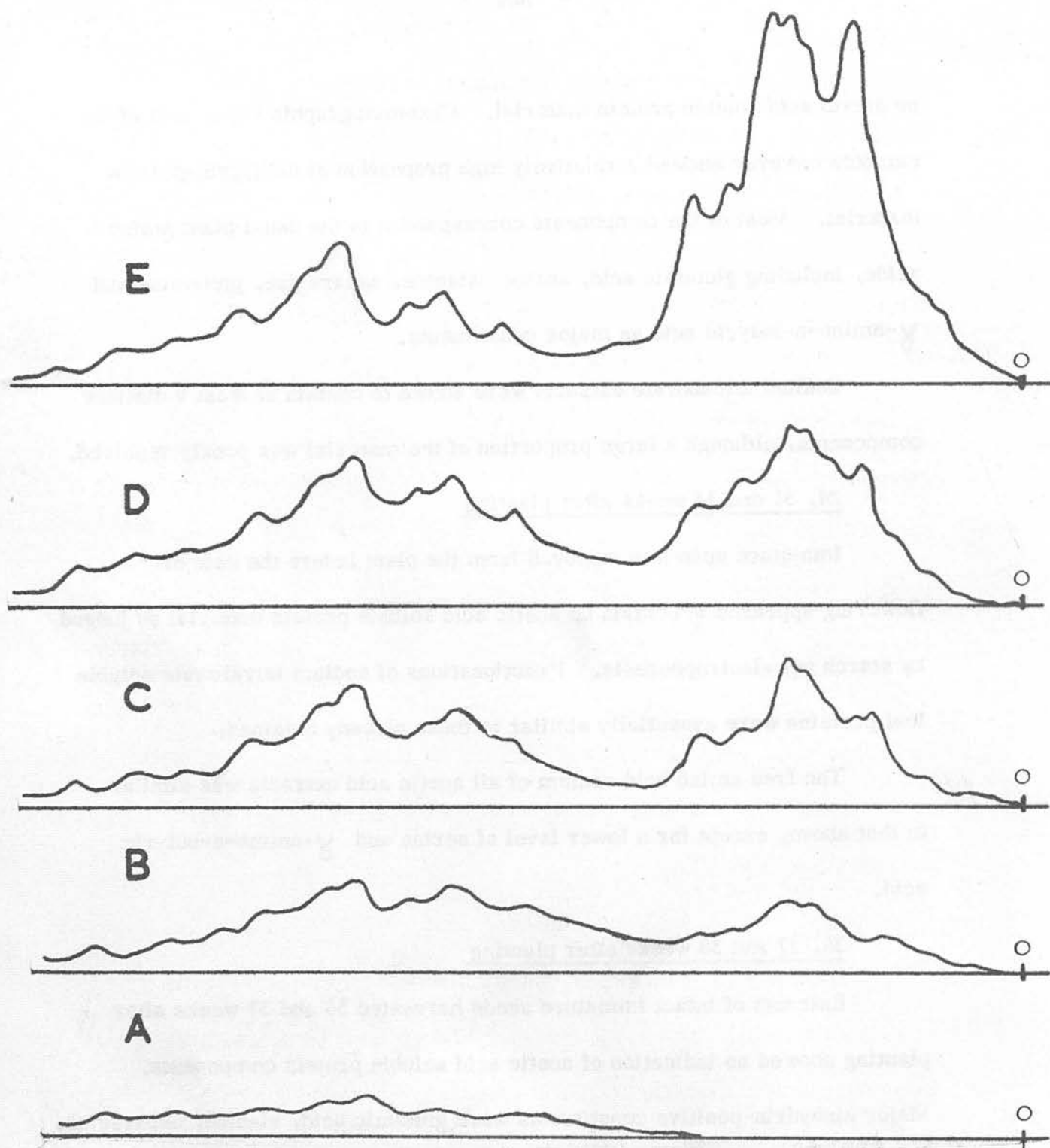


FIG. 19. Starch gel electrophoresis of dilute acetic acid soluble wheat proteins extracted at various stages during ripening.

(A) 38 weeks after planting

(D) 41 weeks after planting

(B) 39 weeks " "

(E) 42 weeks " "

(C) 40 weeks " "

weak, appeared to correspond to components of ripe endosperm of intermediate electrophoretic mobility (Fig. 12, region F). No components of low mobility were observed. Direct chromatographic separations of acetic acid soluble material were unsatisfactory, presumably due to protein interference. Improved results were obtained after ultrafiltration, the major components being similar to those of the previous samples.

39, 40 and 41 weeks after planting.

Acetic acid extracts of endosperm material obtained 39 weeks after planting appeared to contain the full complement of protein components present in similar extracts from ripe seeds. The general distribution of protein material was however somewhat different, the slow-moving fractions (Fig. 12, regions A to D) being extremely weak. A progressive increase in concentration of these fractions occurred after 40 and 41 weeks, while the concentration of the fractions of higher mobility (region F) apparently remained constant. (cf. Fig. 19).

42 to 46 weeks after planting

After 42 weeks, the immature seeds had developed sufficiently to allow removal of the embryonic tissues. This did not however appear to alter the electrophoresis patterns of acetic acid soluble endosperm components. Again an increase in concentration of slow-moving components was observed, with no significant alteration of other fractions.

After 43 weeks little variation in protein pattern was observed,
fractionations/...

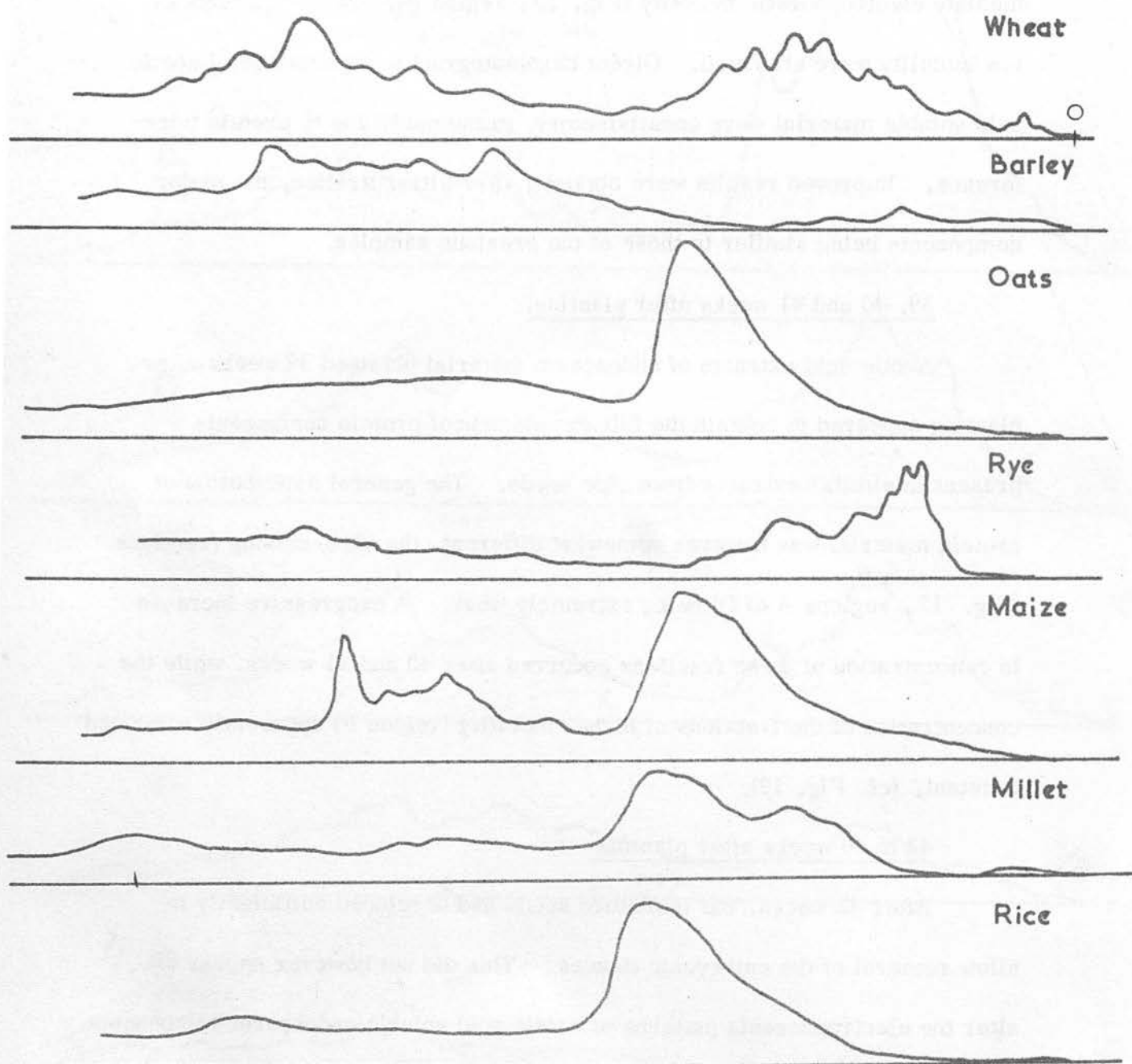


FIG. 20. Starch gel electrophoresis of the water-soluble endosperm proteins of various Graminae.

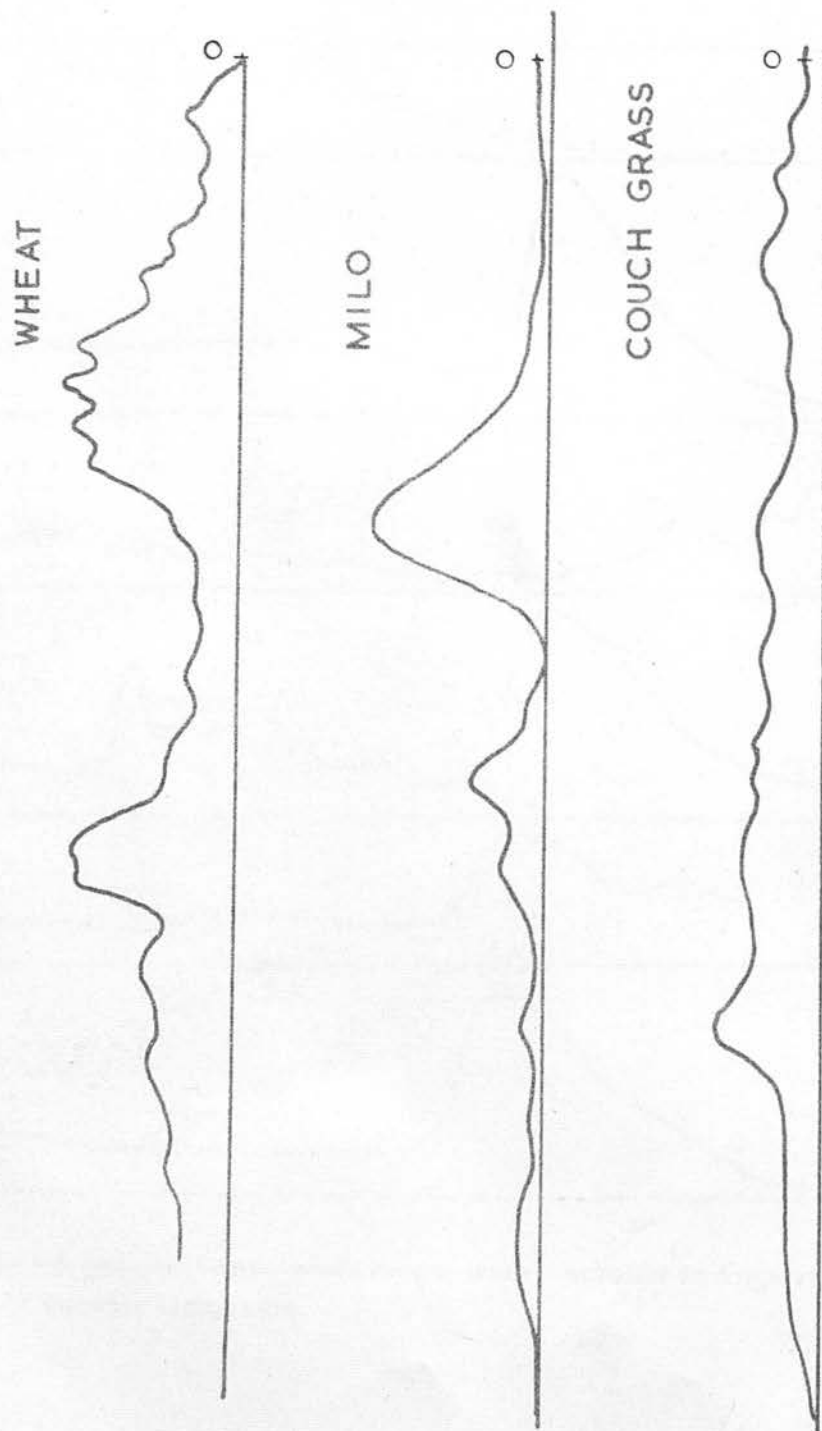


FIG. 21. Starch gel electrophoresis of the water-soluble endosperm proteins of various Graminae.

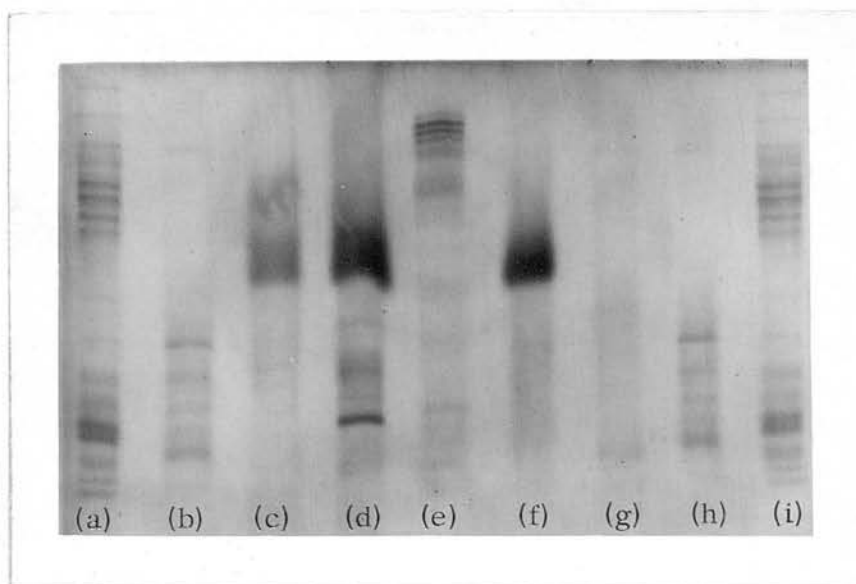


FIG. 22. Starch gel electrophoresis of the water-soluble endosperm proteins of various Graminae.
 (a)(i) wheat, (b)(h) barley, (c) millet, (d) maize,
 (e) rye, (f) oats, (g) couch grass.

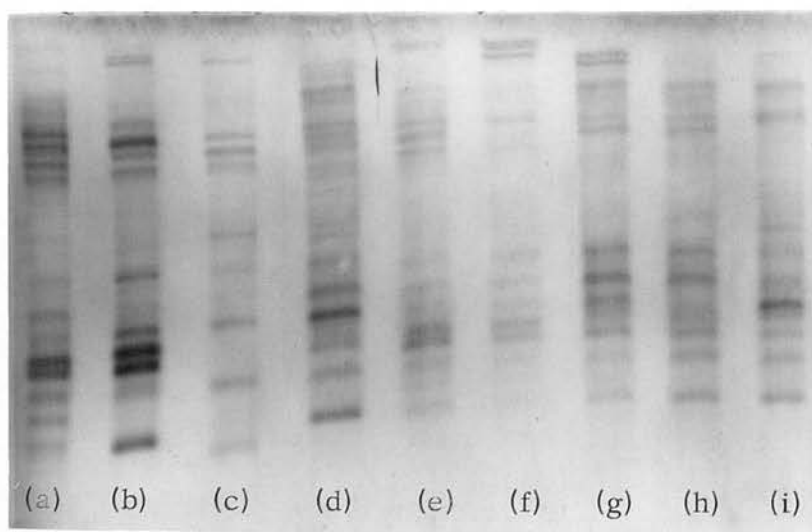


FIG. 23. Starch gel electrophoresis of the water-soluble endosperm proteins of species of *Triticum* and related genera.
 (a) *T. vulgare*, (b) *A. squarrosa*, (c) *T. monococcum*,
 (d) *T. dicoccum*, (e) *T. compactum*, (f) *T. spelta*,
 (g) *T. turgidum*, (h) *T. polonicum*, (i) *T. durum*.

fractionations being identical with those of control samples. No acetic acid soluble protein components were observed in embryonic extracts until after 43 weeks. At this stage only traces of protein material were present. All subsequent samples were, however, identical to control extracts.

(v) Post harvest

No changes were observed in the acetic acid soluble protein fractions of endosperm or embryo material after harvesting.

(c) The protein composition of various Graminae

Aqueous soluble components of all samples were well resolved under the conditions of electrophoresis. Most components, particularly those of barley, maize and sorghum were of relatively high electrophoretic mobility when compared with wheat fractions (cf. Figs. 20 - 22). Millet, rye and couch grass also contained components of this mobility but in much lower concentrations.

Millet, maize, sorghum and rice each contained a major fraction(s) of apparently similar mobility, which was poorly resolved under these conditions.

Few of the extracts, with the exception of wheat and rye, contained components of low electrophoretic mobility. The latter sample contained a large proportion of finely resolved fractions of similar mobility to the slowest-moving wheat components (Fig. 12, regions A to B). Couch grass and barley also contained a small proportion of slow-moving components, the former more closely resembling the wheat protein pattern.

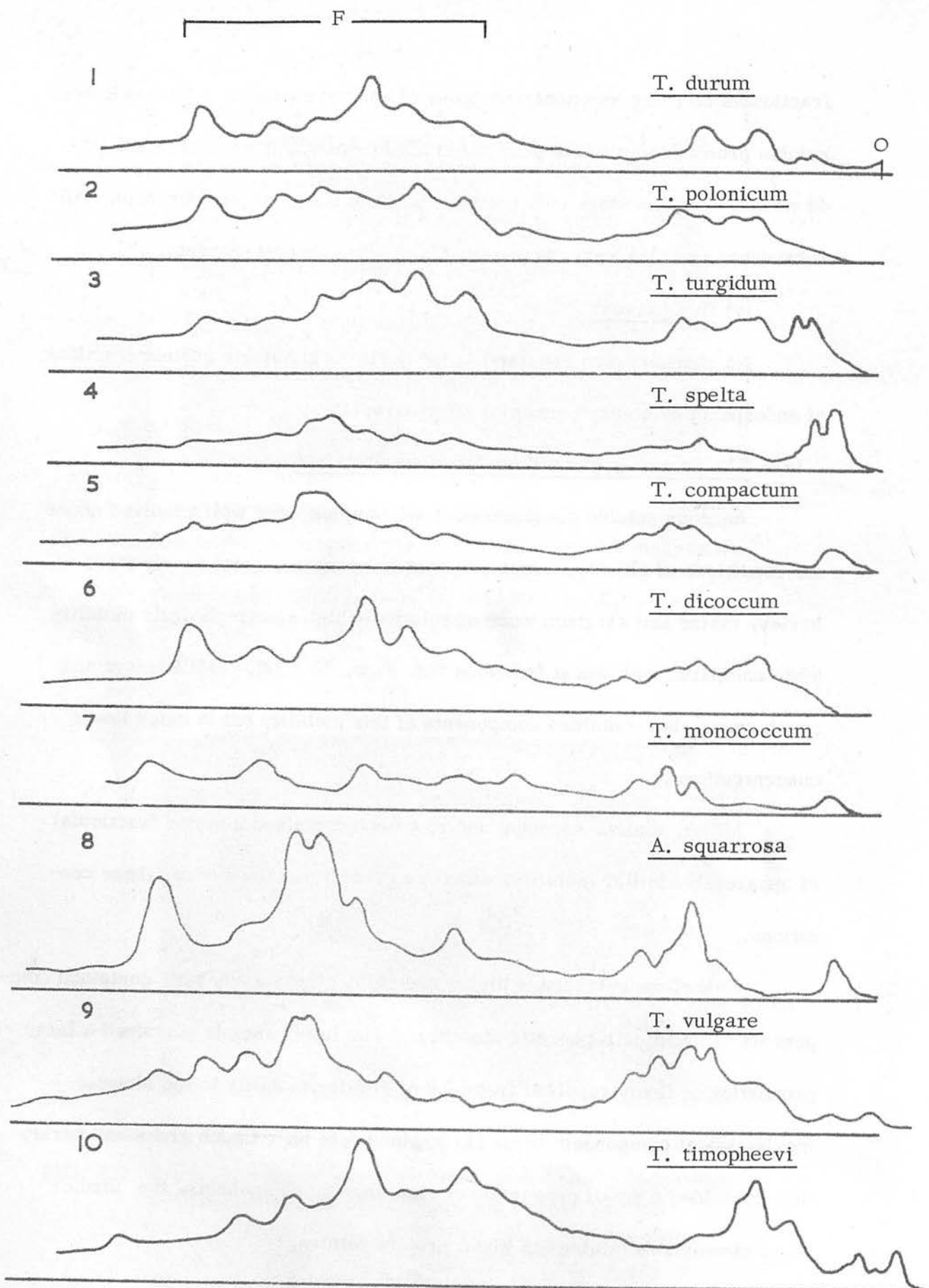


FIG. 24. Starch gel electrophoresis of the water-soluble endosperm proteins of species of *Triticum* and related genera.

(d) Protein composition of species of Triticum and closely related genera

Electrophoresis patterns showed distinct differences in the aqueous soluble protein components of all species (Figs. 23 and 24).

Certain similarities, however, seemed to exist in component distribution particularly in the region of intermediate mobility. These can best be seen from the densitometer tracings in the regions marked F (Fig. 24).

T. dicoccum is very similar to T. durum, while T. timopheevi is somewhat similar, although possessing some unique features. T. turgidum resembles T. polonicum to some extent. All five have rather similar characteristics and can perhaps be classed as one group.

T. spelta is very similar to T. compactum. T. vulgare resembles these two and is somewhat similar to A. squarrosa, although the latter is rather distinctive. These four appear to contain a characteristic doublet which is most marked in A. squarrosa. Generally, they seem to form a second group.

Finally, T. monococcum seems to be unique in that in this region it is unlike any of the other patterns.

Summarising these conclusions.

<u>T. dicoccum</u>	}	very similar	}	Group I		
<u>T. durum</u>						
(<u>T. timopheevi</u>)						
<u>T. turgidum</u>	}	similar				
<u>T. polonicum</u>						

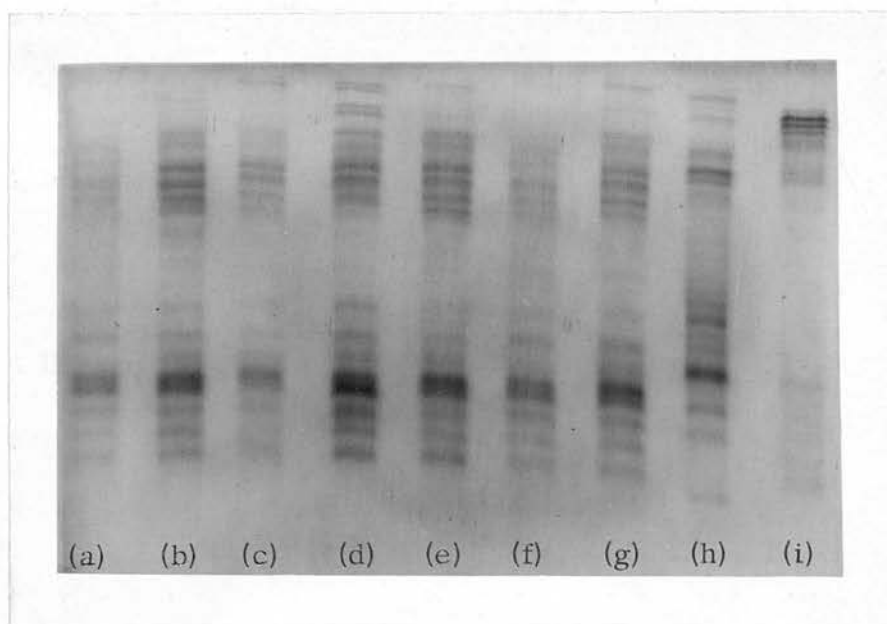


FIG. 25. Starch gel electrophoresis of the water-soluble endosperm proteins of various varieties of *T. vulgare*, *T. timopheevi* and rye.
 (a) Professeur Marchal, (b) Jufy, (c) Capelle, (d) Chieftain x Tenmarq, (e) N59, (f) Professeur Marchal, (g) Als, (h) *T. timopheevi* (cv. IT1), (i) rye (cv. unknown).

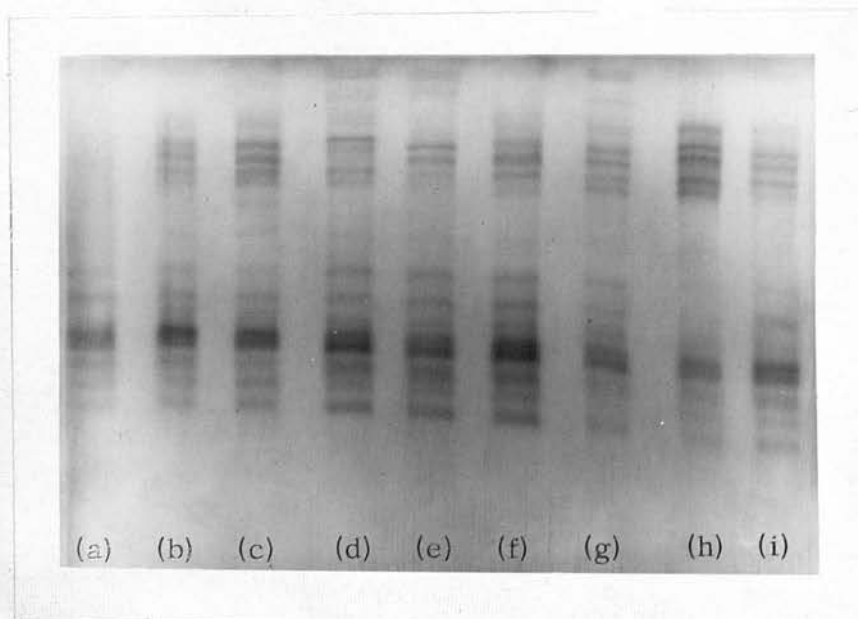


FIG. 26. Starch gel electrophoresis of the water-soluble endosperm proteins of various varieties of *T. vulgare*.
 (a) Professeur Marchal, (b) Capelle, (c) Jufy, (d) Kaw, (e) Manitoba, (f) Russian, (g) Gabo, (h) Champlain, (i) Viking.

<u>T. spelta</u>	}	very similar	}	Group II
<u>T. compactum</u>				
<u>T. vulgare</u>	}	similar		
(<u>A. squarrosa</u>)				
T. monococcum (unique)				Group III

Note: T. timopheevi and A. squarrosa possess some distinctive features not characteristic of their respective group.

Components of low electrophoretic mobility appeared to be somewhat more distinctive of the species, with few similarities in protein distribution. Differences were particularly noticeable among the components of lowest mobility (Fig. 12, region A).

(e) The protein composition of different varieties of *T. vulgare*

Starch gel electrophoresis patterns of the aqueous soluble protein components of various varieties of *T. vulgare* can conveniently be divided according to electrophoretic mobility into a number of groups shown in Fig. 12.

The most distinctive feature of all varieties was the similarity in protein composition of region F within the range of intermediate electrophoretic mobility. All patterns appeared to be identical in this region, showing the same protein distribution observed for *T. vulgare* in the previous section. This pattern would seem to be typical of the species. (cf. Figs. 25, 26).

Distinct differences between varieties were however observed in the region/...

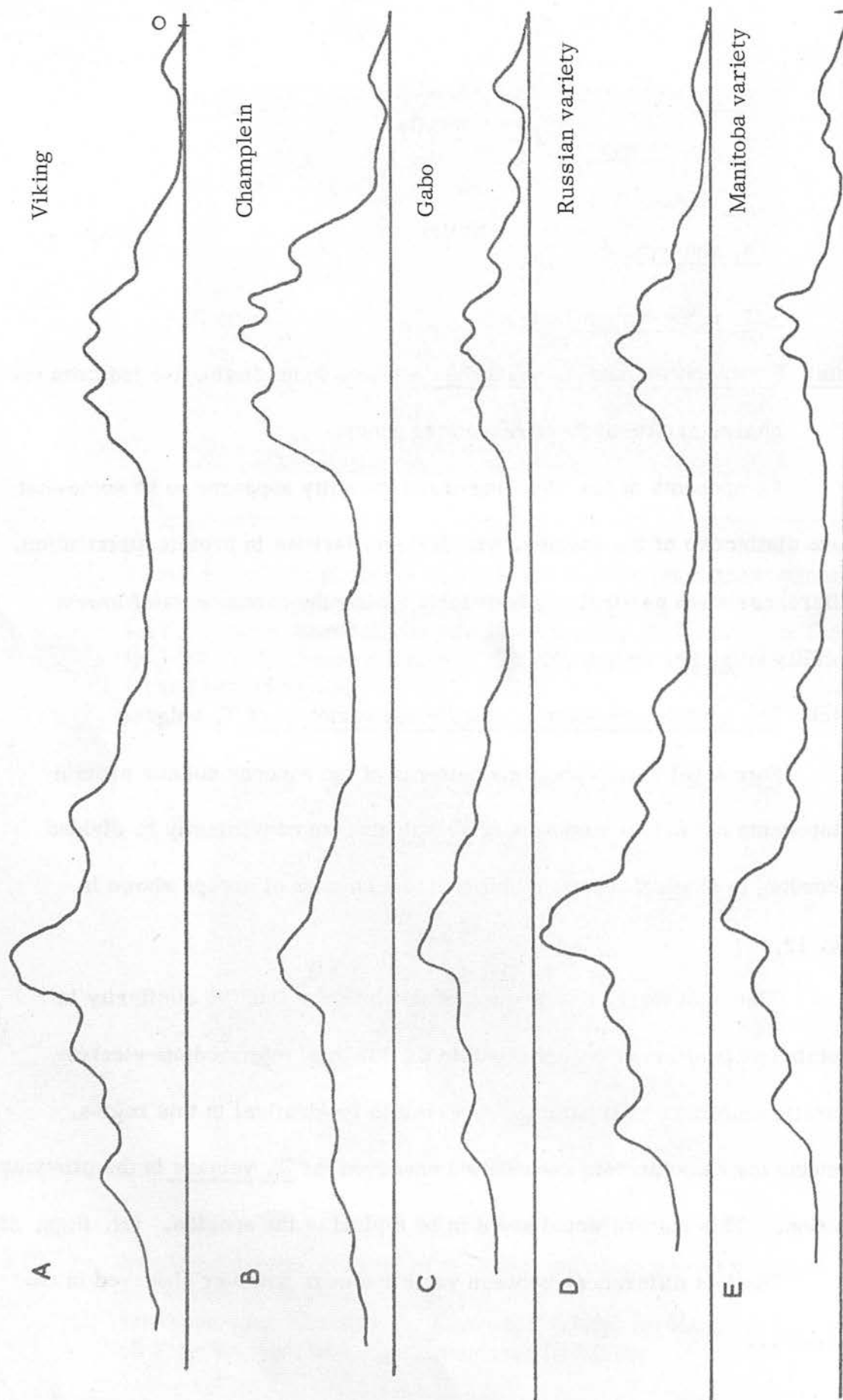


FIG. 27(i). Starch gel electrophoresis of the water-soluble endosperm proteins of *T. vulgare*

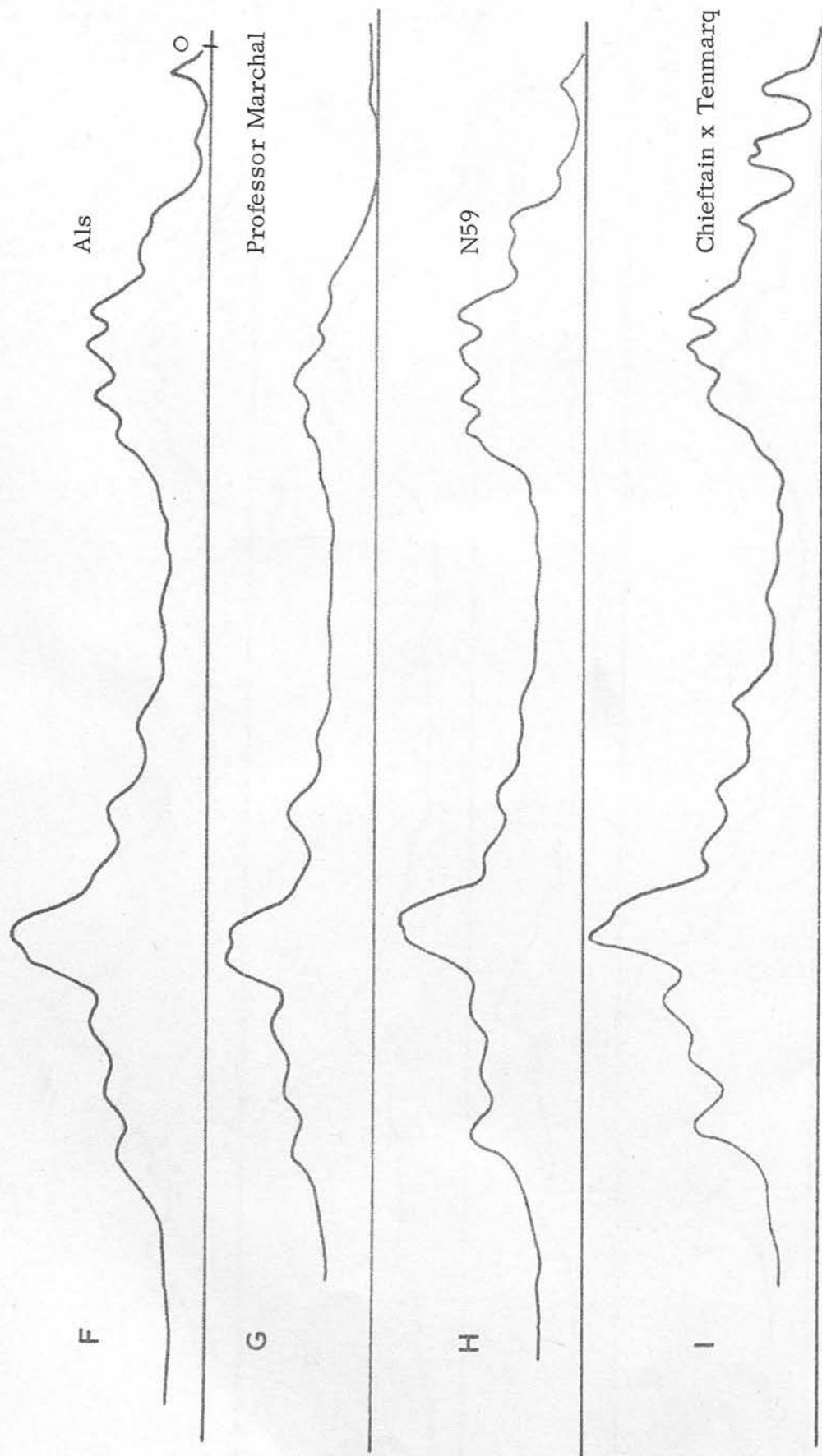


FIG. 27(ii). Starch gel electrophoresis of the water-soluble endosperm proteins of various varieties of *T. vulgare*.

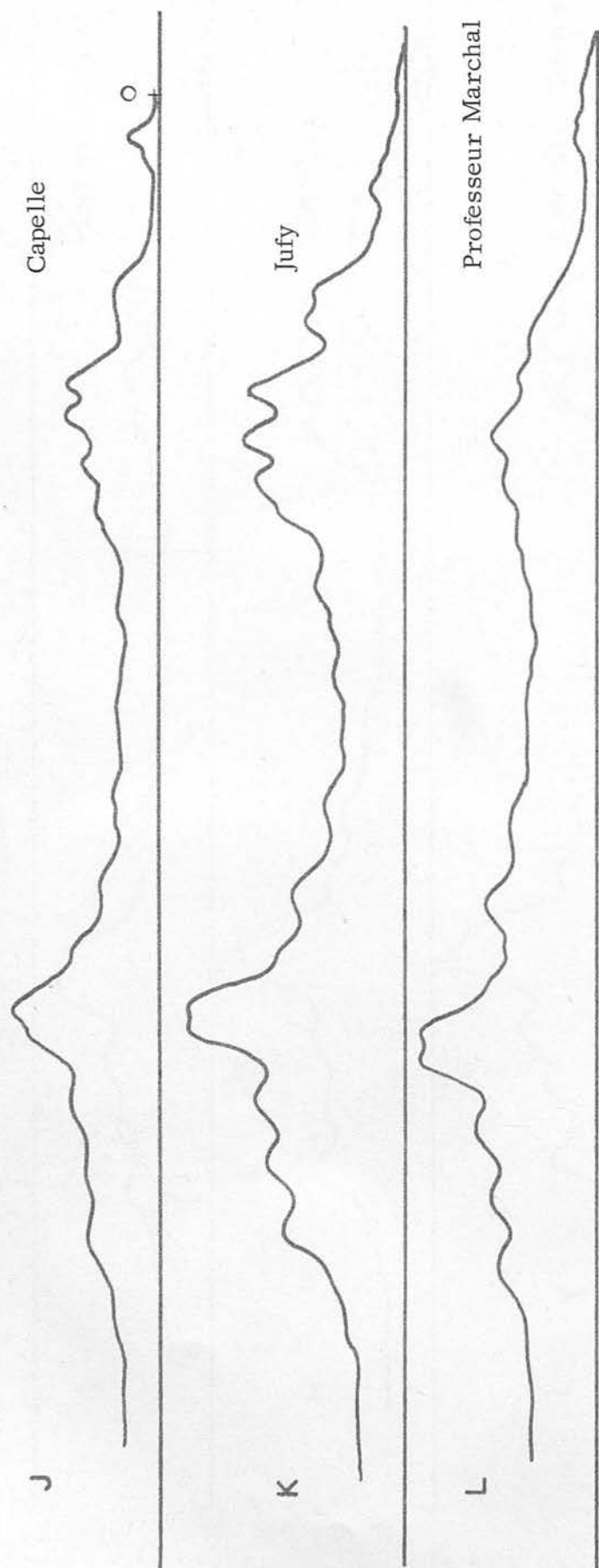


FIG. 27(iii). Starch gel electrophoresis of the water-soluble endosperm proteins of various varieties of *T. vulgare*.

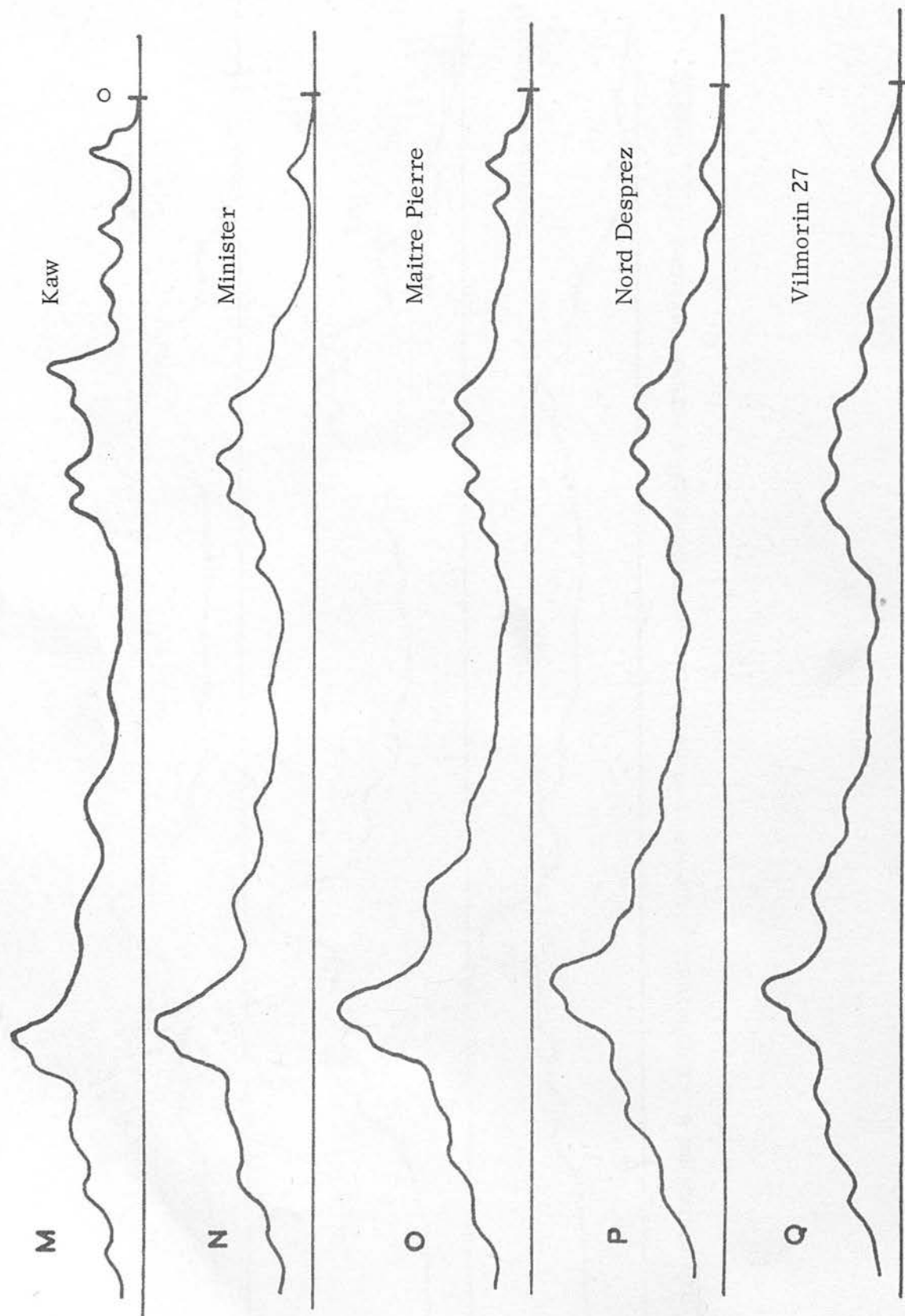


FIG. 27(iv). Starch gel electrophoresis of the water-soluble endosperm proteins of various varieties of T. vulgare.

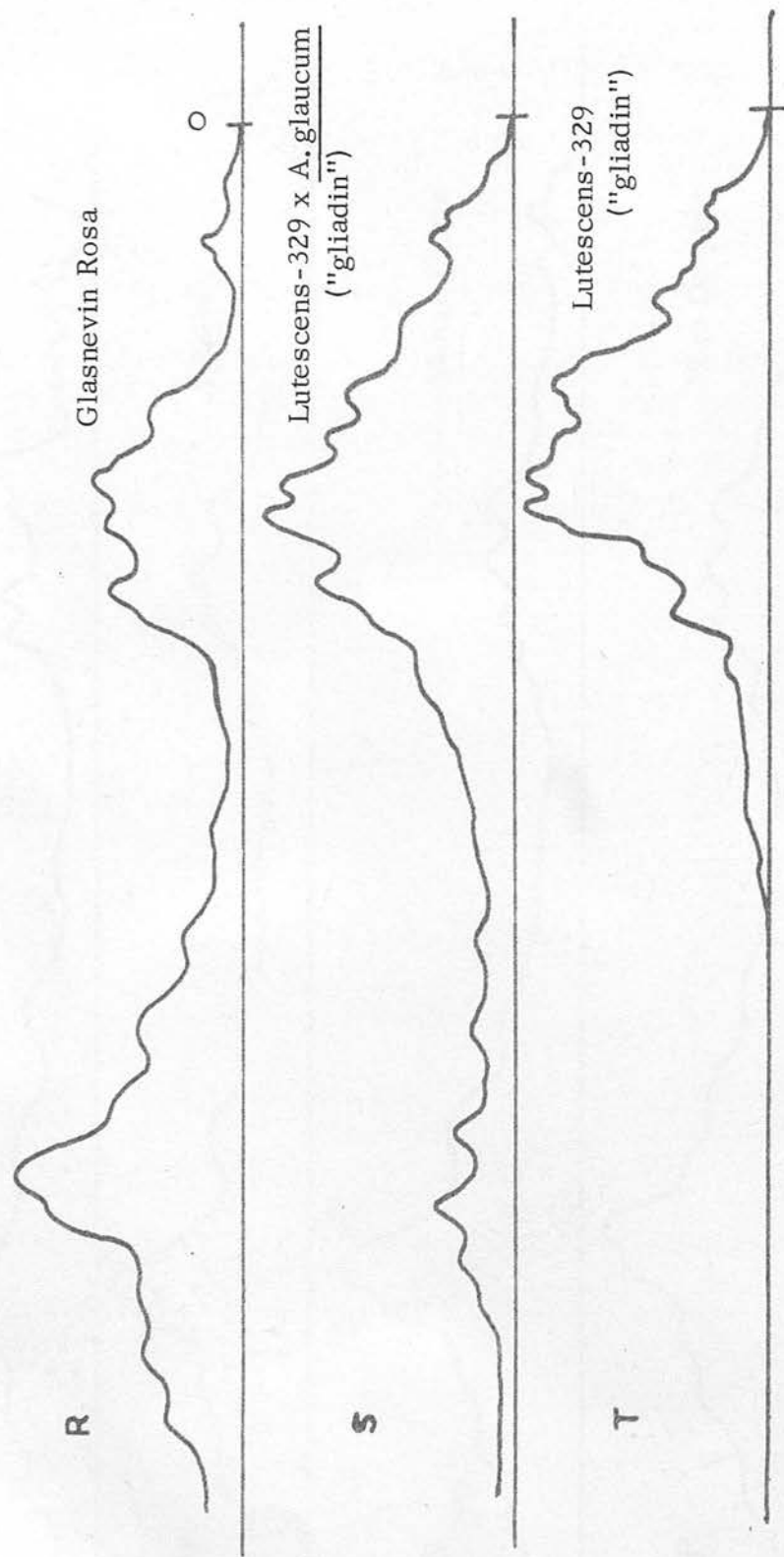


FIG. 27(v). Starch gel electrophoresis of the water-soluble endosperm proteins of various varieties of T. vulgare.

region of low electrophoretic mobility (Fig. 12, regions A to D). These differences were most marked in region A where the number of components varied between 1 and 6. Region C appeared to be similar in many of the samples, although differences in component concentration were observed from the densitometric scans of the patterns. The number of components in region D appeared to vary between 1 and 2.

Minor differences in protein pattern were also observed in region B, but these were essentially of a quantitative nature.

Densitometric scans of protein fractions in starch gel are shown in Fig. 27 (i)-(v).

(f) The protein composition of similar varieties of *T. vulgare* obtained from different sources

Of the samples of *T. vulgare* examined, all varieties which were bred from the same cross but obtained from different sources appeared to contain an identical complement of water-soluble protein components as judged by starch gel electrophoresis. Densitometric scans of protein patterns indicated that the relative concentrations of individual fractions of the same variety were also identical.

(g) The protein composition of related varieties of *T. vulgare*

As described in section (e), the distribution of protein components of intermediate electrophoretic mobility (Fig. 12, region F) was identical for all aqueous extracts.

Distinct/...

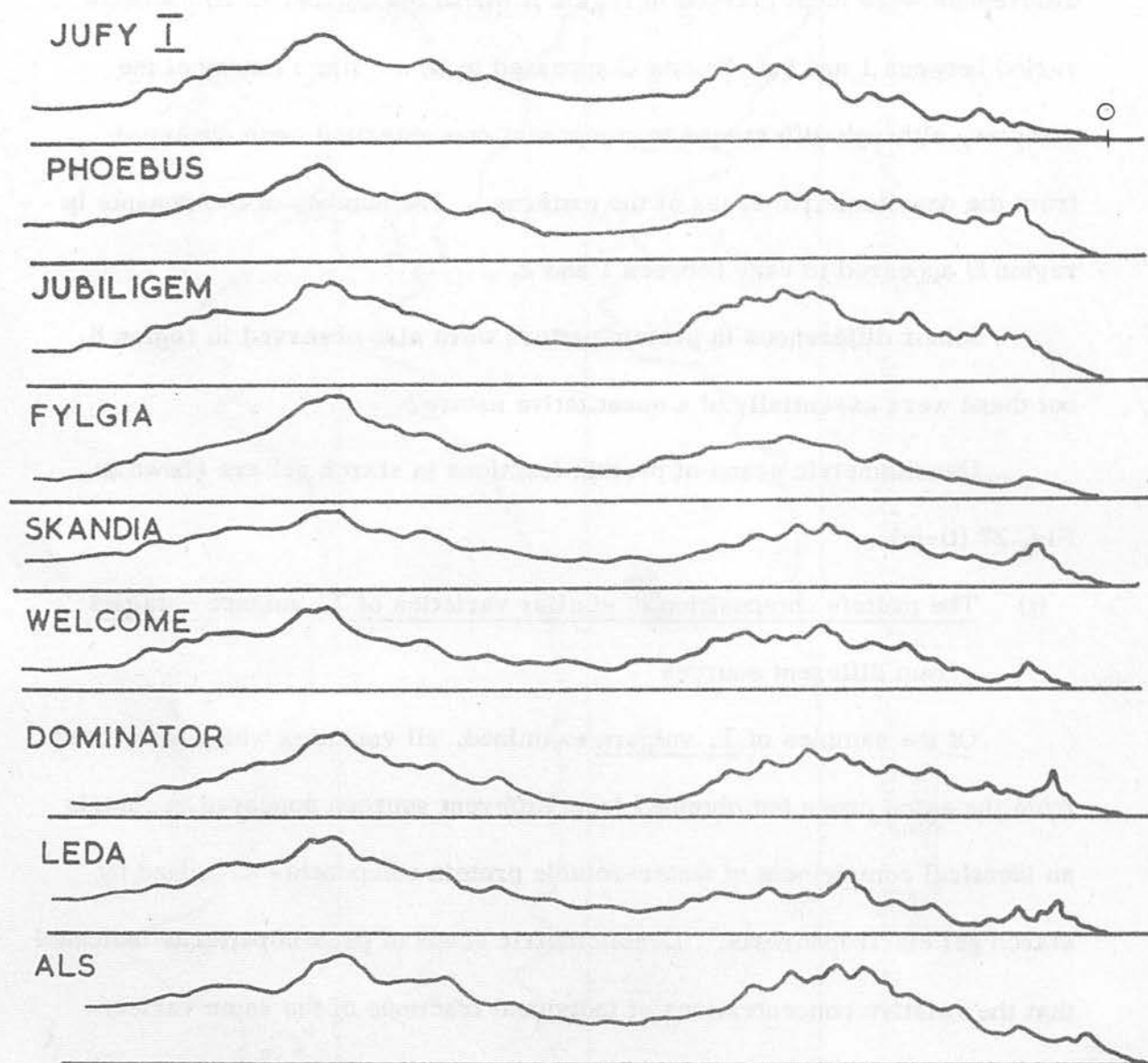


FIG. 28. Starch gel electrophoresis of the water-soluble endosperm proteins of closely related varieties of *T. vulgare* (cf. p. 88).

Distinct differences were however observed in the region of low mobility (Fig. 12, regions A to D), particularly in regions A and D. There appeared to be no obvious similarities in the protein patterns of most closely related varieties (cf. Fig. 28).

In the case of Fylgia and Fylgia II, however, electrophoresis patterns of the water-soluble protein components were identical, although both varieties were known to have originated from separate crosses of the same parents. In contrast, the corresponding patterns, of Jufy I and Phoebus, also obtained from separate crosses of the same parents were distinctly different in the region of low mobility (regions A to D).

(h) The effect of plant selection on protein composition

Aqueous extracts of varieties of T. vulgare obtained from the same cross but developed by plant selection appeared to contain an essentially similar distribution of protein components, as judged by starch gel electrophoresis.

Some minor quantitative differences were, however, observed among the slow-moving components of region B.

DISCUSSION

The method of starch gel electrophoresis has been successfully developed and applied to the fractionation of wheat proteins and other Graminae. This technique has been shown to be superior to other available methods of protein fractionation, presumably due to the molecular sieving characteristics of the starch gel. Fine resolutions revealing up to 32 wheat flour protein components were achieved, while the method, once established, allowed a relatively simple and rapid assessment of qualitative and to some extent quantitative changes in protein composition, in contrast to the more time-consuming classical methods of protein analysis. The detailed resolution also enabled the detection of minor changes in protein composition which would be impossible by other less direct methods. Similar success has also been achieved with other protein systems, evidenced by the fine resolution of serum proteins by Smithies (1955, 1959a, 1959b, 1962).

Recent information suggests that synthetic gels may be even more effective media for protein fractionation, and present results seem to indicate that this method may also be applied with effect to wheat protein systems. The method of preparation of these gels would appear to allow better control over the gel characteristics, making them potentially useful for more detailed fractionations, possibly even on a preparative scale. Their inertness also makes them extremely useful in assessment of enzyme characteristics.

There/...

There seems little doubt that the individual species fractionated by starch gel electrophoresis are of protein character. Isolated fractions have been shown to possess the normal protein characteristics (Elton and Ewart, 1962) showing significant absorption at 280 m μ and producing ninhydrin-positive material on hydrolysis.

Reproducibility of patterns under standardised conditions indicate stability of the system within the terms of electrophoresis, while fractions obtained from Sephadex columns and membrane filtration results also suggest that the stained bands are unlikely to be merely artefacts of electrophoresis. Further evidence of this was obtained by Elton and Ewart (1963) who showed that fractions isolated from starch gels migrated as individual components on re-electrophoresis.

However, these results do not necessarily imply that fractions represent unimolecular species, although the similarity of components extracted in solvents of different dielectric constant seems to suggest that if complexing does occur, something more than mere molecular association is involved.

Molecular sieving experiments through membranes and Sephadex gels indicate the wide range of molecular sizes involved, the fractions of low electrophoretic mobility being extremely large. These fractions may possibly result from complexing of smaller species. Results obtained by ageing protein solutions suggest that the observed increase in concentration of the fast-moving components is connected with the gradual disappearance of the slow-moving/...

slow-moving fractions. Significant precipitation of material was also noted under these conditions, however, but this might be explained in terms of the dissociation of larger fractions into smaller units, some already present in the system, and others being less soluble than the original complex.

Similar effects have also been reported by Cruft (1962a) who observed the dissociation of histones on standing, into smaller molecular species. Moreover, the work of Rowsell and Goad (1962) showed that glutenin could be split by proteolytic enzymes or by reduction, to free a β -amylase fraction (known to be present in the wheat endosperm).

Examination of the protein system during development of the wheat endosperm has shown that the fast-moving components in starch gel are formed some time before the components of low mobility. Whether the smaller fractions represent basic units of the larger components or are perhaps involved as enzyme systems is difficult to establish on the information gained so far. Details of this will however be discussed later.

Examination of the effect of work on the gluten isolate has shown that increased work input results in an increased extraction of the components of low electrophoretic mobility. Axford et al. (1962) have proposed that mechanical action applied to doughs result in extensive -S-S- bond splitting which may possibly lead to the formation of smaller, more soluble components. Under certain circumstances this may also lead to the formation of extra disulphide linkages (cf. Sullivan, 1954) but this would depend/...

depend on the redox conditions involved.

It is the contention of Meredith et al. (1960a, 1960b) (cf. Meredith, 1961) that the gluten complex is an artefact of isolation, which may be split by dilute acid or alcohol treatment. This would seem to be consistent with solubility data discussed later, while shearing forces applied to the complex might also be envisaged to produce similar effects.

The possibility of protein fractions in starch gel being lipo-protein complexes (cf. Olcott and Mecham, 1947; Zentner, 1960) has also been investigated. Treatment of flour with lipid solvents before protein isolation had little obvious effect on the resultant fractionations, indicating that, if lipid material is associated with the fractions, its removal caused no substantial alteration of the protein species. The association of proteins and other substances (e.g. carbohydrates) may also be involved, but this possibility was not investigated here.

The electrophoretic mobility of proteins in starch gel is mainly dependent on a combination of the size and charge characteristics of the migrating species, although shape factors may also be involved to some extent. Sephadex and membrane filtrations of wheat proteins have indicated that protein mobilities in starch gel are largely dependent on molecular size, while density gradient electrophoresis points to a distinct non-uniformity of charge distribution throughout the gel fractions. Certain buoyancy effects, however, may also be involved in the latter case.

Molecular/...

Molecular weight estimations of flour protein components by Sephadex filtrations have indicated that only a small proportion of the fast-moving components are less than 50,000, while some of the components of intermediate mobility as well as most of the slow-moving fractions are probably greater than 100,000. These results are substantiated by evidence obtained from the electrodialysis of protein fractions in starch gel. The degree of penetration of the migrating species through standard membranes, compared with that of proteins of known molecular weight has shown that most of the slow-moving components (Fig. 12, regions A to D) are extremely large, of molecular dimensions apparently greater than 100 m μ .

Some of the slow-moving fractions (region C) however, as well as most of the fractions of region F (Fig. 12) appear to be of molecular weight comparable with that of bovine serum albumin (68,000), while components of higher mobility seemed to be somewhat smaller, but greater than 25,000. These results are based essentially on the electrodialysis characteristics of bovine serum albumin and chymotrypsin and the reported information of Pierce and Free (1961). They must however be treated with distinct reservation, particularly in view of the anomalous behaviour of other standard proteins such as bovine haemoglobin and ovalbumin. General results indicate that although the membranes may be implanted in the gel without danger of electrical disturbance, ionic and steric effects may well influence the passage of certain proteins through the membrane. The uniformity of pore size must/...

must also be considered, but empirical results of Craig et al. (1957) suggest that this is less critical with the type of membrane employed.

The solubility characteristics of wheat flour proteins have been shown to be extremely complex. A refined classification based on solubility would seem to be difficult in the extreme. The classical gliadin, albumin and globulin fractions were all shown to contain significant levels of most of the protein components, while the concentration of individual components was largely dependent on the conditions of extraction. This is in agreement with the work of Pence and Olcott (1952) who found that the solubility of wheat proteins was substantially influenced by salts present in the extracting solutions. Similar results were also reported by Kaminski (1962), Elton and Ewart (1962) and Koenig (1963).

Despite considerable cross-contamination of fractions, certain groups of components were however preferentially soluble in particular solvent systems. Slow-moving fractions (Fig. 12, regions A to D) appeared to be more soluble in aqueous ethanol and to a lesser extent in dilute acetic acid solution, while a higher proportion of the faster-moving components (regions E to H) were extracted in salt solutions. Exhaustive extraction in sodium pyrophosphate solution, however, contrary to the reports of Simmonds and Winzor (1961a), failed to remove completely all of the slow-moving components. Pence et al. (1956) has also reported that even very thorough washing of gluten failed to permit recovery of all "soluble" proteins, and that traces of these components/...

components have been observed in "gliadin" preparations (cf. Woychik et al., 1960). This would seem to suggest an association of these components with the slow-moving fractions • a view also expressed by Dimler and Senti (1959), or might be explained in terms of their production through molecular dissociation as previously discussed.

Similarly, isolated "gliadin" fractions were shown to contain a significant proportion of the faster-moving components, although the bulk of the material was of low mobility. Isolated "glutenin" fractions, however, did not appear to contain any acetic acid soluble components except in one case, where the material of low electrophoretic mobility was totally unresolved. This would seem to substantiate the views of many workers (cf. Blish, 1945) that this glutenin fraction could well represent the denatured residue after alcoholic extraction of gliadin. Moreover, there is certain confirmation of this attitude on studies of barley hordein (Pollock, et al., 1960).

An interesting facet emerged from comparisons of the solubility characteristics of flour and isolated gluten proteins. Aqueous extracts of flour and gluten isolated from an equivalent amount of the same flour, were shown to contain a very different proportion of protein components (Fig. 13). The latter extract contained a far greater amount of the slow-moving fractions with the exception of those of lowest mobility in group A (cf. Fig. 12). This seemed to suggest the involvement of the gluten complex formation, but subsequent examination of aqueous extracts of doughs aged for various periods gave/...

gave protein patterns identical with those of flour. It would thus appear that the final step of gluten isolation, viz. starch removal after gluten formation, may play an important part in the extractability of these fractions of low mobility.

Hess (1952, 1954) reported the presence of two morphologically distinct types of protein material in flour, viz. wedge and adhering protein, the latter being closely associated with the starch granules. He postulated that a combination of these two fractions was necessary for formation of the gluten complex (cf. Meredith et al., 1960a, 1960b).

Electrophoretic examination of samples of wedge and adhering protein obtained from Professor Hess' laboratory have shown that these fractions indeed contain a very different complement of protein components (Fig. 17). Wedge proteins contained only slow-moving fractions (Fig. 12, regions B to D), while adhering proteins contained fractions of very low mobility (region A) together with some of the fractions of higher mobility (regions E and F). The slow-moving adhering protein components were not necessarily identical with other flour fractions of a similar mobility since some variability of the fractions in this region did occur, depending on the type of flour used. Nevertheless, it would appear that a distinct group of protein components are associated with the starch granules. The removal of starch after dough formation may well be an important step in the complete formation of the gluten isolate.

Examination/...

Examination of the aqueous extracts of air-classified flour fractions of different protein content showed no distinct differences in component composition. The method of flour fractionation, based essentially on the findings of Hess (cf. Jones et al. (1959a) is presumably much less complete when carried out on a commercial scale. Similar results have been reported by Jones and Dimler (1962) and Wrigley (1963), although amino acid analyses of Stevens et al. (1963) have suggested that proteins of the gliadin-glutenin type are extracted in flour fractions of small particle size.

The starch gel electrophoresis method was also applied to the assessment of materials of different rheological characteristics. Distinct differences were observed in the protein components of glutens of different character, particularly among the slow-moving fractions, but no correlation could be obtained between protein composition and gluten quality. The differences observed were probably indicative of differences in wheat varieties used, as will be shown later.

Examination of samples of weak, medium and strong flours showed similar minor differences in protein composition which appeared to be unrelated to their rheological characteristics. These results are not however surprising, since rheological characteristics may well involve constituents other than proteins, e.g. lipids and carbohydrates. Moreover, proteins in solution are hardly likely to be truly representative of the three-dimensional gluten gel complex as such.

Wheat/...

Wheat proteins in solution have been shown to possess a considerable stability in contrast to many other protein systems. Fractions were relatively unaffected by dispersion in urea or by heating for short periods at 98-100°C. This was also observed by Jones et al. (1959b) and Woychik et al. (1961b). In contrast, the isolated gluten complex was readily denatured by drying at elevated temperatures (>100°C). It is possible, however, that minor alterations of protein components might not be observed under the conditions employed, since loss of enzyme activity, e.g. β -amylase has been shown to occur after heating for short periods.

Similarly, ultrasonic treatment and relatively high levels of γ -irradiation (8 K rad) appeared to cause no alteration of the protein components although higher levels of irradiation (35.6×10^6 rad) were shown to result in extensive alteration of the protein system. A large proportion of the material still remained soluble, however, but was not fractionated under the conditions of electrophoresis. Patterns were essentially similar to those of certain glutenin extracts, suggesting that this loss of resolution may be indicative of at least partial denaturation of the protein material.

Various chemical treatments which are known to cause significant alteration of the rheological properties of flour doughs and isolated gluten appeared to have little affect on the protein components in solution. The inclusion of small amounts of oxidising and reducing agents in doughs, following industrial procedures, produced no detectable protein alteration as far as could/...

could be judged by starch gel electrophoresis. Although rheological changes may not necessarily be reflected by changes in the proteins in solution, these effects could possibly be explained in terms of oxidation and reduction of other non-protein systems closely connected with dough and gluten rheology. Tsen and Hlynka (1962) have proposed an alternative oxidative pathway whereby the lipids compete with free sulphydryl groups of proteins for available oxygen. Narayanan and Hlynka (1962) have also shown that doughs from defatted flour are more resistant to oxidation effects indicating the probable redox participation of lipids in the gluten complex.

Moreover, removal of lipids from flour, while causing a drastic reduction in gluten-forming capacity, has been shown to leave the protein system essentially unaltered. Similarly, significant loss of visco-elasticity of gluten by phospholipase action was not reflected in terms of protein alteration.

Consequently, more marked effects brought about by prolonged oxidation and reduction of flours may not be truly representative of industrial improving procedures. It is interesting however to note that oxidation under these conditions led to an apparent decreased solubility of protein components. This could possibly be explained in terms of increased intermolecular disulphide bonding as proposed by Sullivan (1954) with the formation of larger, less soluble species. In contrast, prolonged reduction appeared to increase the solubility of protein components and was accompanied by the formation of new apparently smaller molecular weight components. These effects, akin to/...

to proteinase action, might be at least partly explained in terms of proteinase activation as proposed by Jørgensen (1935a, 1935b), although the more popular view of reduction of disulphide bonds must also be considered (cf. Bloksma, 1958).

It should be noted, however, that both oxidation and reduction effects are more marked among the components of low electrophoretic mobility (Fig. 12, regions A to D).

The involvement of these slow-moving fractions was also noted on examination of enzyme effects on gluten proteins. Papain/cysteine systems were shown to bring about marked degradation of these fractions, without significant alteration of the components of higher mobility (Fig. 12, region F). The protein fractions, however, appeared to be surprisingly stable to trypsin and pepsin attack, possibly due to the conditions and levels employed or to the presence of inhibitors in the system. The presence of trypsin inhibitors in flour have been reported by Learmonth and Wood (1960). Moreover, wheat is known to contain papain-like enzymes (cf. Jørgensen, 1935a, 1935b) and this may possibly explain the sensitivity of the system to papain attack.

Attempts to differentiate gluten proteins in terms of peripheral peptides from partial hydrolysates were relatively unsuccessful under the conditions employed. Although very different "fingerprints" were obtained from different glutes, the reproducibility of patterns was not sufficiently good to justify utilisation of the method without more detailed studies. The complexity/...

complexity of the protein components could yield undetected minor variations in protein extracts (some have been observed by starch gel electrophoresis) and could easily lead to significant variations in peptide "fingerprints". The method is undoubtedly more suited to systems of relatively few protein components.

Studies on the proteins of wheat throughout the life cycle of the plant produced some interesting results, although no drastic changes, except perhaps in the case of embryonic proteins, were observed. Results obtained indicated the progressive degradation and utilisation of storage protein material during germination and its gradual resynthesis during ripening.

Analysis for total nitrogen (dry weight basis) of germinating wheat endosperm appeared to indicate the rapid utilisation of protein material during the 4 to 5 days of germination, compared with that of other constituents, e.g. starch. This is presumably indicative of protein degradation and translocation in view of the relatively low levels of non-protein nitrogen in the dormant wheat endosperm. The results presented in Fig. 18 could also be explained in terms of slower rates of utilisation of starch, and other components, over the initial period which increases after the 5th or 6th day.

This rapid removal of nitrogen-containing material was not however detected by starch gel electrophoresis of endosperm proteins until after the 6th day of germination. Over the period from the 6th to the 9th day, components of low electrophoretic mobility (Fig. 12, regions A to D) become progressively/...

progressively weaker presumably due to protein degradation and translocation of the nitrogen-containing material. It is of interest to note that, although the degradation pattern resembled to some extent that produced by proteolysis, no significant corresponding build-up of new components of smaller molecular weight was observed. This would seem to indicate, as expected, a rapid utilisation of the degraded material.

In contrast, components of intermediate electrophoretic mobility (Fig. 12, region F) remained essentially unaltered throughout this period. Similar patterns were obtained up to the 13th day, after which these components became progressively weaker until the 17th day when only traces remained. A similar degradation pattern was observed by Danielsson (1951b) in pea seeds. The breakdown of reserve proteins was shown to be greatest from 5 to 10 days after germination, while albumin proteins appeared to be degraded at a slower, more constant rate. Similarly, Bagley *et al.* (1963) reported the major period of protein alteration in germinating peanuts to occur between the 4th and 9th days, while most of the storage protein material appeared to have been utilised by the 15th day.

From these results, it would appear that the components of low electrophoretic mobility are more intimately involved in the germination procedure and possibly represent the true storage proteins of the endosperm. Components of higher mobility may well represent some of the enzyme systems of the plant which are involved in the metabolism of storage material. This conclusion/...

conclusion is further substantiated by evidence from the ripening process discussed below.

Embryonic proteins, which have been shown to be quite distinct from endosperm components, appear to perform a very different role during germination. These components were shown to disappear quite dramatically during the very early stages, to be replaced by components of different solubility characteristics. The normal complement of acetic acid soluble protein components present in the dormant embryo was observed only during the 1st and 2nd days of germination. After this period only components soluble in solutions of higher pH, e.g. sodium tetraborate, could be extracted. This would seem to imply the involvement of these acetic acid soluble components in one of the first stages in the germination process, perhaps as initiators of other processes which lead to endosperm protein degradation. A more detailed examination of these components, particularly during vernalisation, would be of interest.

The synthesis of acetic acid soluble protein components of ripening wheat endosperm seems to substantiate further the view that the components of low electrophoretic mobility may represent the storage proteins of the dormant grain. It was of interest to note that these components were only produced at detectable levels very late in the ripening process. Acetic acid soluble fractions were observed only for the last 5 weeks before final ripening of the grain (cf. Fig. 19). At this stage only components of intermediate electrophoretic mobility/...

mobility (Fig. 12, region F) were observed. The concentration of these components, as judged by staining intensities, appeared to remain relatively constant throughout subsequent stages, in contrast to the progressive build-up of slower-moving fractions (Fig. 12, regions A to D). After the first appearance of fractions of low electrophoretic mobility, no further qualitative changes in protein composition were observed, since each extract appeared to contain the full complement of protein components present in the ripe endosperm. Similar quantitative changes were reported by Graham *et al.* (1962). Danielsson (1952) also reported a complete and rapid formation of reserve proteins of pea seeds during ripening. This was taken to imply the rapid synthesis of protein by way of large molecular weight materials rather than a gradual build-up from the available amino acids.

Accurate assessment of the appearance of acetic acid soluble embryonic components was extremely difficult due to the small amounts of material available. Results suggest, however, that these fractions also appear towards the end of the ripening period.

Examination of the water-soluble endosperm components of different Graminae has shown distinct differences in protein composition. Samples of each genus, some of unknown variety, were examined. These patterns may not be truly representative of all species of a particular genus, but in view of later investigations of wheat species, it seems likely that they will give a good representation of the general protein distribution.

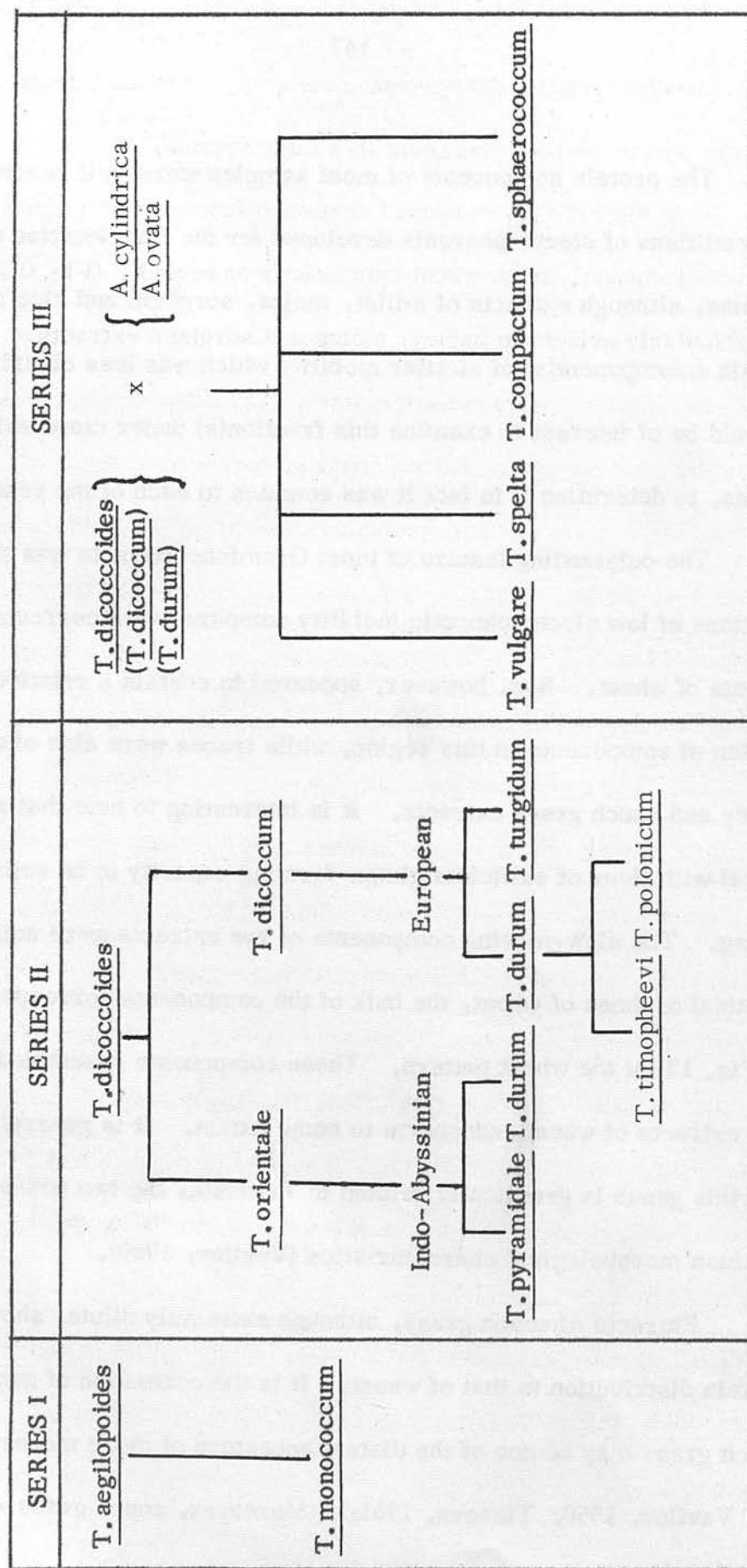
The/...

The protein components of most samples were well resolved under the conditions of electrophoresis developed for the fractionation of wheat proteins, although extracts of millet, maize, sorghum and rice appeared to contain a component(s) of similar mobility which was less clearly resolved. It would be of interest to examine this fraction(s) under more suitable conditions, to determine if in fact it was common to each of the genera.

The outstanding feature of most Graminae extracts was the lack of fractions of low electrophoretic mobility compared with corresponding components of wheat. Rye, however, appeared to contain a relatively high proportion of components in this region, while traces were also observed in barley and couch grass extracts. It is interesting to note that rye is the only cereal with flour of sufficient gluten-forming capacity to be suitable for bread baking. The slow-moving components of rye extracts were not, however, identical to those of wheat, the bulk of the components corresponding to region A (Fig. 12) of the wheat pattern. These components resembled adhering protein extracts of wheat endosperm to some extent. It is generally accepted that this genus is genetically related to *Triticum*, the two possessing many common morphological characteristics (Vavilov, 1950).

Extracts of couch grass, although extremely dilute, showed a similar protein distribution to that of wheat. It is the contention of geneticists that couch grass may be one of the distant ancestors of more modern wheats (cf. Vavilov, 1950; Tiunova, 1961a). Moreover, couch grass-wheat hybrids have/...

FIG. 29. Genetical relationships between wheats (Percival, 1921).



have been shown to contain the normal complement of wheat components.

This aspect will be further discussed in a later section.

Most of the samples examined contained fractions of a mobility, similar although not identical, to the wheat components of region F (Fig. 12). These were particularly evident in barley, maize and sorghum extracts. The protein distribution of the latter samples was remarkably similar, which may possibly indicate some genetical relationship between the two.

An electrophoretic examination of the water-soluble protein components of various wheat species and genetically related genera has shown that certain distinct qualitative and quantitative differences exist in protein composition. Densitometric scans of these patterns (Fig. 24) have shown, however, that certain similarities exist between the proteins of different species, particularly in the region of intermediate mobility (region F). By comparison of these results with the proposed genetical table (Fig. 29) it can be seen that these fractions appear to reflect to a large extent the genetical characteristics of the species. Although only single varieties of each species have been examined, it seems likely that the components of this region are typical, if not common, for all varieties of one species. This view is supported by subsequent patterns obtained from several varieties of T. vulgare (cf. Figs. 27(i)-(v)).

It is interesting to note that the species of Aegilops squarrosa and Triticum monococcum are by far the most distinctive, and these possess both distinctive/...

distinctive morphological and genetical characteristics when compared with other wheat species. The most pronounced feature of A. squarrosa is a characteristic protein doublet in the starch gel, which is also present, but less marked, in T. vulgare, T. spelta and T. compactum. The latter species are considered to have been developed through cross-breeding between Aegilops and the tetraploid species (Fig. 29, Series II) so it would seem conceivable that this distinctive doublet may be a reflection of the Aegilops characteristics.

Although couch grass is considered by some to be related to present day wheats, protein fractionations unfortunately did not merit comment of this nature. Protein patterns obtained using protein isolate from a wheat-couch grass hybrid, however, appeared to suggest that components of intermediate mobility were not identical with those of the parental wheat (cf. Fig. 27(v)). This has also been reported by Tiunova (1961a) who used a fractional precipitation procedure. As only gliadin preparations of this sample were available, the components of this region were distinctly weak and so did not permit a more accurate evaluation of the protein pattern. It would be of interest therefore to examine available wheat hybrids, particularly those of the diploid, tetraploid and hexaploid species (Fig. 29, Series I, II and III).

Although different species contained a very different complement of components of low electrophoretic mobility, the patterns in this region did not/...

not appear to be typical of the species (Figs. 23, 24).

Examination of several varieties of T. vulgare has also shown that distinct differences exist in protein composition in this region (Figs. 25-27). These differences were mainly confined to the fastest (A) and slowest (D) fractions, particularly the former. It is interesting to note that region A may correspond to those components associated with the starch granules (adhering protein).

Although it is not certain whether the slow-moving components (regions A to D) are characteristic of the variety, identical patterns were obtained from the same variety supplied from different sources.

These varieties were, however, all grown in Scotland, under presumably similar environmental conditions and it is possible that very different climatic and soil conditions may cause significant alteration of the protein pattern. It seemed justifiable however to investigate further the nature of these components since all other available varieties examined were also grown under rather similar conditions.

Several related varieties of T. vulgare were shown to contain an identical complement of protein components of intermediate mobility (Fig. 12, region F) typical of the species, while distinct differences were again observed among the components of low mobility (regions A to D). No correlation could however be obtained between these components and the corresponding components of the parental forms (cf. Fig. 28).

It/...

It was noted, however, that two varieties, Jufy I and Phoebus, bred from the same parents but obtained from separate crosses contained significantly different slow-moving protein components. A second pair, Fylgia I and Fylgia II also of the same parental forms but different crosses, contained essentially similar components. Although different crosses would be expected to produce different varieties, it is interesting to note that the last two varieties also possess distinct morphological similarities - presumably the reason for retaining the name in the latter cross. This would seem to imply some possible relationship between certain protein components and morphological characteristics, although these similarities may well be purely coincidental.

The protein components of varieties obtained from the same cross but developed through plant selections showed distinct similarities, with minor differences in two of the slow-moving components.

From the present investigation it would seem likely that genetical influences play an important role in determining a large part of the protein composition of the wheat endosperm. Minor variations probably arise through environmental effects but their significance and magnitude require further investigation.

Since preparation of this report, a series of papers have been published concerning the changes in protein components of developing wheat endosperm (cf. Jennings and Morton, 1963a, 1963b, 1963c; Graham, 1963; Graham et al., 1963a, 1963b/...

1963b; Graham and Morton, 1963; Jennings et al., 1963).

Since many of the methods employed in the above investigations were similar to those reported here, it is of interest to review the results in the light of present findings. The major difference in the two reports would seem to be in the time factor involved for the ripening process. Although the precise date of wheat ripening is not given, the period from flowering to ripening in Australia would appear to be approximately 33-40 days. The corresponding period in this study was approximately 67 days.

By following the changes in dilute acetic acid and sodium pyrophosphate soluble proteins throughout the ripening process, it was shown that from the 19th day after flowering until maturity, there was a rapid increase in the amount of acetic acid soluble endosperm proteins (slow-moving fractions in starch gel) compared with pyrophosphate soluble proteins (intermediate mobility) (Jennings and Morton, 1963a). This would seem to be in agreement with the present results, since similar observations were made from the 31st day after flowering (39 weeks after planting) (cf. Fig. 19) which is roughly equivalent on the Australian time scale.

No protein components of low electrophoretic mobility were observed in pyrophosphate extracts of their initial field sample (13 days after flowering), in contrast to all subsequent extracts. They did however detect a small amount of these components in dilute acetic acid extracts. This date of sampling roughly corresponds in the present study, to the date of first appearance/...

appearance of protein fractions of intermediate mobility (cf. p. 83), when no slow-moving fractions were observed. The differences in these observations may however be due to their selection of stages: Graham et al. (1963b) did not examine any stages before the 13th day.

Jennings and Morton (1963), by comparison of protein/ribonucleic acid in acetic acid and pyrophosphate soluble proteins throughout ripening, concluded that the pyrophosphate soluble components may be intermediates in the formation of the acetic acid soluble fractions. This, however, could not be confirmed by the radioactive tracer experiments of Graham and Morton (1963).

SUMMARY AND CONCLUSIONS

1. A method of starch gel electrophoresis has been developed for the fractionation of wheat and related protein systems which allowed the resolution of up to 32 wheat protein components.
2. By using this technique to separate wheat protein fractions obtained by column electrophoresis in a density gradient, and by examination of the effects of solvents of different dielectric constant, it has been possible to establish with some certainty the validity of the fractions as separate, but not necessarily unimolecular, species. The involvement of protein-protein complexing has been suggested from solubility data.
3. Membrane filters and gel molecular sieves (Sephadex) have been used to obtain estimates of the molecular size of these protein components. A very wide molecular weight range was indicated.
4. Examination of the solubility characteristics of wheat proteins has shown that a proper classification cannot readily be made on this basis. Protein solubility depended largely on the conditions of extraction and may possibly be influenced by the gluten complex formation.
5. Starch gel electrophoresis has been used to study the effects of physical, chemical and biochemical factors on the wheat protein system.

The proteins have been shown to possess considerable stability, being unaffected by heating, certain denaturing agents, ultrasonic treatment and γ -irradiation/...

γ -irradiation (8 K rad). Higher levels of γ -irradiation (35.6×10^6 rad), however, led to extensive protein alteration.

The inclusion of oxidising and reducing agents in doughs following industrial improving procedures, had no detectable effect on the protein system, while more prolonged treatment led to an apparent reduced solubility on oxidation and marked protein alteration on reduction.

Rheological changes in gluten, brought about by lipid removal or phospholipase treatment were not reflected in terms of protein alteration, indicating the probable redox participation of lipids in gluten.

Wheat proteins were readily degraded by papain attack, but were less sensitive to tryptic and peptic action. This may be due to the presence of inhibitors in the system.

6. Examination of flours and glutes of different physical characteristics has shown distinct differences in protein composition, but these could not be correlated with rheological data.

7. Wedge and adhering proteins isolated from wheat endosperm have been shown to contain a very different complement of protein components, indicating, with solubility data, probable protein-starch interactions. Air-classified flours similarly examined showed no distinct differences in protein composition.

8. The changes in the wheat protein system during the life cycle of the plant have been followed using the starch gel electrophoresis technique.

Fractions of low electrophoretic mobility were progressively degraded during/...

during germination and progressively synthesised towards the end of the ripening period, indicating their probable role as storage proteins of the endosperm. In contrast, fractions of higher mobility were less affected during germination and were utilised at a later stage. Similarly, these components were synthesised first during ripening and remained at a relatively constant level during the build-up of apparently high molecular weight material. On the information available it was not possible to determine whether the fast-moving fractions represented the enzymes of the system or were precursors of larger components.

9. The endosperm proteins of various Graminae have been examined. Pronounced differences in protein composition were shown, the general distribution giving some indication of the relationship of the genus to wheat.

10. A comparison of the endosperm proteins of different wheat species and closely related genera has shown that certain protein fractions probably reflect the genetical characteristics of the species. Certain similarities in protein composition have been correlated with available genetical history.

11. A study of the endosperm proteins of several varieties of T. vulgare has shown distinct differences in the composition of fractions of low electrophoretic mobility. Although certain of these components may be typical of the variety, others may be influenced to some extent by environmental factors.

FUTURE STUDIES

In view of the complexity of the wheat protein system, a natural follow-up to starch gel fractionation would seem to be the isolation of individual components, possibly by development of column chromatographic techniques or by the use of synthetic gels on a preparative scale.

This would allow a more detailed study of such factors as enzyme characteristics and protein-lipid, protein-carbohydrate interactions. In this context, a more detailed investigation of wedge and adhering proteins as isolated by Hess would seem profitable, particularly in their relationship to the isolated gluten complex.

Further study of the protein changes during the wheat life cycle would seem necessary to determine the relationship between storage and other proteins, as well as the nature and role of embryonic proteins in the seed. In view of the unsatisfactory classification of wheat proteins on a solubility basis, a better classification might be achieved in terms of biochemical function. A histochemical study combined with antigen-antibody precipitation techniques using fluorescent antibodies, might be of value here.

A detailed study of various Graminae and in particular crosses between different species would allow more comment on the present findings.

The relationship of wheat protein components to extremes of climatic and fertiliser conditions would lead to a better understanding of the protein composition/...

composition. The possibility of identification of wheat varieties (which is at present extremely difficult) and the evaluation of new varieties and inheritable factors of quality, would be extremely useful to the wheat breeder.

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APPENDIX I

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(17) Starch-Gel Electrophoresis of Isolated Wheat Gluten

By C. B. COULSON and A. K. SIM. (*Arthur D. Little Research Institute, Inveresk, Midlothian*)

Isolated wheat gluten was shown by Elton & Ewart (1960) to consist of at least eight components when fractionated by starch-gel electrophoresis [standard gel but aluminium lactate buffer, Jones, Taylor & Senti (1959), Naphthalene Black Stain]. We find that by concentrating with Carbowax 20M (G. T. Gurr Ltd.), reducing the concentration of starch in the gel (to 10%, Connaught Starch-Hydrolysed; 8-10 v/cm. for approximately 2 hr.) and cutting extensive colour loss by staining in Nigrosine (Smithies, 1959), more than twenty components can be observed.

After washing the flour in butanol and pyrophosphate solution, Simmonds & Winzor (1961) fractionated the acetic acid extract on a carboxymethylcellulose column into six components. The presence of an additional two components was attributed to incomplete removal of aqueous-salt-soluble 'non-gluten' proteins (Simmonds & Winzor, 1961; cf. Pence, Mecham & Olcott, 1956). Proteins were isolated from wheat flour by the Simmonds & Winzor (1961) procedure. On examination by starch-gel electrophoresis we find the 2M-sodium pyrophosphate solution (pH 7) only partly extracts several components, mainly of an intermediate mobility. On standing at 4° for several days, changes occurred in the concentrations of the gluten fractions dispersed in 0.1N-acetic acid, although the overall qualitative pattern remained constant. The faster-moving components became more concentrated at the expense of those of lower

mobility. This may be due to protein dissociation perhaps partly into the genetic protein units, with the establishment of a new equilibrium, in which case the pyrophosphate-soluble 'non-gluten' components may include some of the basic components of gluten. Ethanol-soluble gliadin (British Drug Houses Ltd) was found to correspond to the slower-moving fractions—in agreement with the free boundary electrophoresis findings of Jones *et al.* (1959).

The gluten components, present in acetic acid solutions, do not appear to succumb readily to denaturing agents, unlike most proteins. Short heating times (95°; 3 min.) do not appear to alter their solubility or their mobility in starch gel. The addition of urea (to about 8M) to the protein solution before electrophoresis does not cause any change in the patterns observed. Some proteins are known to regain their native state when the urea-containing solution is diluted (Kauzmann, 1959).

The changes in gluten constituents on standing suggest that even greater changes take place when gluten is dispersed. Yet gluten in solution is relatively difficult to denature by heating and probably only suffers reversible denaturing in urea.

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APPENDIX II

Abstract from Proc. 1st Int. Congr. Fd Sci. and Tech., 1962, p. 10.

Biochemistry and Biophysics of Isolated Wheat Gluten by C.B. Coulson, A.K. Sim and Elspeth A. Somerville, Arthur D. Little Research Institute, Inveresk, Scotland, U.K. Starch gel electrophoresis (pH 3.1, aluminium lactate buffer, $\mu = 0.009$) used in conjunction with Nigrosine stain and using 10% starch (Connaught starch-hydrolysed) concentration at 8-10 V/cm yields a pattern of 28 protein bands for N/100 acetic acid soluble gluten. Further studies using this improved technique indicate that fast-moving low-molecular weight bands are not "contaminants" and that "gliadin" has six components which fall into the slow-moving higher molecular weight group. Paper chromatographic studies on phospholipids of isolated gluten indicate a complexity similar to that of flour. A Rhodamine 3G0 method for rapid estimation of phospholipids, suitable for use with column chromatography, is given.